Medical Lib.

VOLUME VIII NUMBER 3

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MAY, 1923

EDITOR-IN-CHIEF

C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

PUBLISHED BI-MONTHLY
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

Entered as second-class matter April 11, 1918, at the postoffice at Baltimore, Maryland, under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917. Authorized on July 13, 1918

Copyright, 1923, Williams & Wilkins Company

Price, net postpaid { $8.00 per volume, United States, Mexico, Cuba, Canada
$8.50 per volume, other countries

Made in United States of America

PEPTON

Perfectly serviceable for the formulas and in all the technic of the bacteriological and antitoxin laboratory. It is employed in the usual proportions and for whatever purposes Pepton of this most desirable quality is required.

Manufactured by

Fairchild Bros. & Foster

New York

"The Sun Never Sets on"

PFANSTIEHL SUGARS

THESE carbohydrates because of their quality have become the STANDARD FOR BACTERIOLOGY in every country in the world. The Pfanstiehl list has been extended until it now comprises 70 of the rare carbohydrates, in addition to such other rare reagents as Amino Acids and certain Blood Test Solutions.

We shall take pleasure in sending on request the list of Pfanstiehl Chemicals, or a reprint of "The Rare Sugars: Their Purity and Tests" (Pfanstiehl and Black).

Specify Standard of Pfanstiehl

SPECIAL CHEMICALS COMPANY

DEVOTED TO THE NATIONAL INDEPENDENCE OF AMERICA

HIGHLAND PARK, ILL.

WHAT CONSTITUTES EFFICIENCY IN RESEARCH?[1]

L. A. ROGERS

Research Laboratories of the Dairy Division, United States Department of Agriculture

In selecting this subject I realized that it was not a new one, and that nearly everything that could be said on the topic had already been said in one way or another. If I had known then as well as I know now how very much had been said, how many presidential addresses on the organization of science fill the pages of *Science*, I think that I would have had compassion and spared my audience this last straw. And yet, in going through these numerous addresses and letters, I cannot help thinking that in some cases the writers have failed to distinguish between the real organization and the defects of the organization, between the meaning of the word "efficiency" as used in business, and what constitutes efficiency in research. I use the words "organization" and "efficiency" deliberately, realizing fully the extent to which they have been overworked and the antipathy which they arouse in the minds of some efficient directors of well organized laboratories.

One does not need to read many pages of the journals in which scientists exchange ideas to realize how many look upon an organization as applied to research as something in part, if not wholly, evil. For them the word "organization" is associated with supervision, with administrative duties, with everything that is the antithesis of the freedom of thought and action assumed to be essential to real research.

We are frequently reminded that many of the great men of science have worked alone and the inference is drawn that their accomplishment was great because they were unhampered by

[1] Address of the President at the Twenty-fourth annual meeting of the Society of American Bacteriologists, Detroit, Michigan, December, 1922.

an organization. Someone has painted an amusing picture of Newton ordered by the director of a modern organization to make a progress report on his theory of gravitation. There seems to be a fear in some quarters that if one is truly scientific he cannot be "efficient." This aversion to efficiency has no doubt been accentuated by the association of the word in the past few years with a systematized effort to increase the output of machines and manual labor. From this we have come to associate the word "efficiency" with stopwatch studies, with close supervision, with time cards, and with detailed daily reports. Organization and efficiency remind us of the proposal, seriously made during the war by a prominent American, for a research institution surrounded by a high brick wall and manned by a force of scientists working in eight-hour shifts. We are afraid that in an effort to increase our efficiency, our freedom of working out our problems in our own way, and at our own convenience will be curtailed.

It must be admitted that some of the fears incited by the word organization have a basis in fact. It is true that many of our ablest investigators have ceased to be producers through the encroachment of administrative duties incident to a growing organization. The preëminent scientists of the past—the Newtons, Darwins and Pasteurs were not forced to pay this penalty for proved ability, and continued to be producing investigators throughout their lives. We can estimate now what the world would probably have lost if these men had been obliged to devote the greater part of their energies to administrative work. If Pasteur had been the director of a modern laboratory, preparing reports and estimates, attending meetings of committees and research councils, speaking at dinners and women's clubs, would his name have become so interwoven with our daily life that its use may be taken as a measure of the state of civilization of a people? But little can be proved one way or the other by what did happen, or what might have happened to the Newtons and Pasteurs; they were extraordinary men and would have risen above the distracting influences of an organization just as they rose above the lack of material equipment.

Certain evils have become so associated in our minds with a large organization that we are inclined to look on these evils as inherent and unavoidable. Red tape is not confined exclusively to the business of the government, but may be found entangling the work and impeding progress in any large organization. It is safe to say that the greatest difficulties which the average investigator has to overcome are not involved in his problem itself, but are those thrown in his way by man-made organizations. Usually these obstacles are constructed in the name of efficiency and by those who are employed to assist, not to obstruct.

I could cite from my own experience many instances which illustrate how a highly complex organization frequently defeats the end for which it was created and these, no doubt, could be matched by any one connected with any large institution. In the course of an investigation to which a vacuum pump was essential the shaft of the pump was broken. Now, an inefficient scientist, unhampered by a highly organized supply system, would have sent a messenger to the nearest hardware shop for 2 feet of 1-inch steel and in a few hours the machinist could have made a new shaft; but a purchasing committee which, by its own confession, is highly efficient, had protected the financial interests of the government by contracting with a dealer in Pittsburgh, for a year's supply of this particular kind and size of steel. Consequently, it became necessary to make a formal request for a requisition on this dealer, a process which in the normal course of events requires several days, and then to wait the pleasure of the dealer in filling this large order. Fortunately, in this case, the purchasing committee had not foreseen the possibility of needing parts for this particular kind of pump and we had the privilege of ordering a new shaft from the manufacturer. By using the telegraph it was possible to reduce the delay to a few days.

Many of the complications of organization have resulted from the application of the popular fallacy that efficiency and economy can be promoted by the consolidation of small units into larger ones. One of the inherent evils of a large organization is the tendency of the clerical element to dominate the scientific force. As the organization grows the worker becomes further removed

from the source of authority, the paths through which the business of the organization must pass become devious and full of obstacles, and the hands of the worker become tied by regulations and precedents. The economy of consolidation is also more frequently apparent than real. If the function of a library is merely to distribute books at the least possible expense, no doubt this end could be best accomplished in a centralized library, but it is better to appear extravagant by spending money for branch libraries than to have a laboratory force work for weeks or months on a problem because, through difficulty in getting books and periodicals, they are ignorant of the fact that the problem has been solved.

One of the windmills against which the organizers most frequently tilt is the duplication of work. It is easy to see that in certain types of work duplication does become a great evil, but in research work it is not duplication but the lack of duplication that is to be avoided. Few investigations lead to wholly correct conclusions and new results become established as facts only when they are confirmed by several workers. It is only by the cumulative effect of disconnected attacks on a problem from different angles that progress is made. This may be true even in a single laboratory in so far as it involves application of different types of mind to the same problem.

Evidently the evil of which the writers of the presidential addresses stand in greatest fear is a curtailment of the investigator's inherent right to develop his own ideas in his way.

Dr. A. Franklin Shull (1), speaking of ways of discouraging or limiting freedom of research says that "one of these ways is the appointment of an investigator to a position for the purpose of studying a certain problem." He cites a terrible example where this actually occurred and adds, "By the terms of his appointment his energy could be directed into other channels only with the permission of his superior officer. Such direction from above could be justified only in the case of an assistant or an investigator on temporary appointment, not in the case of a permanent colleague."

I must confess to many transgressions along exactly this line, and for my peace of mind would like to have someone tell me how the very definite problems which confront us can be solved without thus limiting freedom of research. The money with which the investigator's salary is paid, and his equipment purchased, may have been provided for the study of a very definite question and it does not seem unreasonable to expect him to stick to his last. The particular method by which he attempts to solve the problem is another question, but even here many beginners are unfortunate in having had too much freedom. Some men are successful only because, throughout their career, a skilful pilot has held them to the course.

William Morton Wheeler (2) after a very entertaining description of the characteristics and habits of the genus scientific investigator, concludes that "Attempts at organizing investigators must fail because their highly specialized activities depend to such a great extent on their peculiar native aptitudes or capacities."

While I must admit some evidence of temperament, my experience has led me to believe that investigators do not differ in their essential characteristics from other people.

It is true that the qualities which make a successful investigator are different from those which make a successful salesman, or financier, or mechanic, but are we ready to admit that they preclude the possibilities of systemization or coöperation?

However, the trend of the presidential addresses is not entirely toward individualism as opposed to organization. Milliken (3) says, "The extraordinary rapidity with which scientific developments were made during the war was unquestionably due then, first, to the forming of these highly competent research groups; and, second, to the establishment of effective channels for the coöperation between these groups."

This process of organization and coöperation, while greatly stimulated by war conditions, is not an especially new movement.

Less than a quarter of a century has seen the development of great institutions with resources, equipment and organization undreamed of by our fathers. We have voluntarily formed what

has been described as a "superorganization of superorganizers," the National Research Council. We are told that we must coöperate. The efficiency of existing organizations is to be increased by coördination and consolidation. The problem of organization is continually with us whether we welcome it or not. No laboratory is so small as to avoid it. When an investigator acquires an assistant it comes to him. When a man becomes an assistant he faces an organization problem and his is, perhaps, the more difficult one because he lacks the authority to put his ideas into effect.

In building up our organization there are certain obvious ends to be gained and certain obvious evils to be avoided. We perfect our organization to obtain efficiency. Our funds are usually provided for definite purposes. We have a program of work planned, a particular end to attain, and the money available should be so expended that it will carry us the greatest possible distance on the road to that end. Of even greater importance is the necessity of expending the energy and ability of the workers so that they will yield the greatest returns in the advancement of human knowledge.

The danger in any organization of scientific work lies in the tendency to submerge the individuality of the worker. In such organization we are not dealing with machines, nor with piece workers. In research work the unit of the organization is a highly developed human mind. The product which our organization turns out is the result of the thought of an individual worker, and just so far as the organization inhibits or distracts these minds from their true course it is inefficient. On the other hand, the organization promotes efficiency so far as it tends to permit and to stimulate originality and freedom of thought in the individual worker and at the same time to coördinate and concentrate the activities of the several investigators on the particular problem in hand.

In considering means of attaining this end we must keep in mind the distinction between the methods of the factory efficiency engineer and those which must prevail in the laboratory. In the factory the road to efficiency leads through system, routine,

supervision, coördination of men and machines, office records and elimination of unnecessary motions. In the laboratory efficiency is obtained by reducing supervision to the lowest point compatible with the ability of the investigator; by removing him from the distractions of report writing and routine office work; by surrounding him with an atmosphere conducive to study and meditation; by providing him with the special equipment necessary to the solution of his problem and by promoting a spirit of coöperation which will unite the individual investigators into a compact body working together on a single group of problems. We should always keep in mind that the staff is the laboratory not the building or the equipment which are frequently exhibited as examples of a great laboratory. Liebig is said to have done some of his best work in a kitchen; Pasteur, working in a dark basement was directing a great laboratory.

However, in building our organization on the individual worker we must keep in mind that there are diverse kinds and conditions of men. Scientific investigators may be divided into two classes, the leaders and the followers. By "followers" I intend no disparagement of the great class of scientists to which most of us belong. Indeed, it would be very difficult to decide which is of the greatest value. Daniel Boone and his fellow explorers who blazed the way through the mountains and the pathless forests into the Kentucky valleys made a great state possible but without the settlers who followed in their footsteps and turned the wilderness into productive farms and thriving cities their efforts would have been valueless and forgotten.

The leaders are the exceptional men, with imagination, who have the faculty of grasping the significance of phenomena that to the ordinary worker seem trivial or incomprehensible, and who, by a series of brilliantly planned experiments, push back a little further the wall of darkness that marks the limit of human knowledge. For a man of this type the organization must afford wide latitude in the selection of problems and in the method of solving them. On the other hand, there are many men who, if left to the dictates of their own imagination, flit from one unfinished project to another and accomplish little beyond their

own entertainment, while they may, under the right leadership, become good investigators. For these men a more rigid project system is necessary. For the great majority of investigators who depend for their progress on industry, perseverance and logical thinking all that is necessary is the essential equipment and an opportunity to develop their particular problem.

In developing an organization consideration must also be given to the type of work to be done, although the underlying principles apply to all cases. The only real difference in research institutions is in the primary object to be gained. Is the institution designed to produce investigators or the results of investigation? Each will train men and each will increase knowledge, but the organization and methods of work will necessarily be along different lines. The classification into laboratories for pure science as contrasted with applied or industrial laboratories is not basic but merely indicates the scope of the laboratory. In the laboratory of pure science knowledge is sought for knowledge's sake. The laboratory is in a way incomplete, a fragment of a laboratory. The investigator, through inclination, lack of facilities or appreciation of the possible application of his results fails to carry his investigation to the point at which the value is evident.

In the words of Huxley "What people call applied science is nothing but the application of pure science to particular classes of problems." The applied laboratory which does not develop its own problems in pure science must begin where others have left off and carry the investigation on to its application. The more highly organized of the industrial laboratories have provided for what we will continue to call, for lack of a better term, pure science, wisely permitting great latitude in the selection and development of problems. The wisdom of this course is already evident in the fundamental results of great scientific and economic value which have come from some of the investigations of these laboratories. No one can foretell the results of investigations of this nature or estimate their value, not even their pecuniary value to the company which profits by the investigation. The history of the progress of science in the past teaches us that the great

discoveries of the future, will be along lines which cannot be conceived in advance. Discoveries, which to the ordinary layman are the creatures of a single master mind, are the natural sequences of a long series of investigations. They are not isolated points, but the apexes of pyramids shining in the sun of publicity. Few, if any, of these investigations were undertaken with even a hope that their application would benefit humanity. The invaluable x-ray was only an incidental result in a series of investigations on the nature of the electric discharge. These studies were made possible by the perfection of methods of obtaining high vacua and this in turn was developed to permit the determination of the atomic weight of thallium. The public knows of the "discovery" of x-rays, but is not interested in the nature of the electric discharge and knows, and cares, nothing about the atomic weight of thallium.

The research laboratories of the universities are usually organized on a somewhat different basis from the industrial laboratory in that the main function is teaching and the results of research are incidental. The limitation of many of the investigators both in time and experience, and the necessity of selecting problems which will give training in research to successive generations of students, narrow the scope and determine very largely the nature of the organization.

In an industrial laboratory, while there is frequently necessity for training new men, the organization is based entirely on research for the sake of the results.

A complete research organization has four functions: (*a*) The study and development of new laws and fundamental truths without regard to their application or usefulness; (*b*) the development of possible uses for new knowledge, and the solution of problems which arise in the varied activities of mankind; (*c*) the trial of results obtained in the laboratory under conditions which will test their applicability to practice; (*d*) the dissemination of the results obtained. Few organizations are complete in this sense. Some confine themselves to the study of fundamental science only; some build on the basic facts developed by others; few have the facilities for applying what they learn under working conditions.

It is very easy to make diagrams on paper showing how, in a logical way, this should be done, but diagrams, no matter how well done, cannot really represent human beings, and as Mr. Little has observed, "There is danger in an organization chart—danger that it may be mistaken for the organization." Whatever type of organization is chosen, the executive functions will center about the administrative head of the institution. We are in the habit of designating this official as a director, although the term does not fit harmoniously with our conception of scientific research. The motive power for research must come from within and it can be directed only in a very limited way. The head of a research organization must be, not a director but a leader. His function is not so much to direct research as to recruit a staff; to provide the necessary material equipment; to maintain an atmosphere conducive to constant application, and to coördinate the work of the investigators on the problems of the laboratory. The members of the staff are his associates, not his assistants, and he serves the ends of science best when he stands between them and the distractions of correspondence, finances, and report writing.

The form of organization which has been most used in the past follows the lines of the basic sciences. This requires departments of chemistry, bacteriology, pathology, or whatever sciences may be involved. It necessitates arrangement for coöperation between departments on any problem involving more than one science, something which is very easy to show in a diagram, but very difficult to obtain in practice. This system has the advantage of making it possible to have an especially strong man responsible for the work in each branch of science, but carried to its logical end establishes artificial barriers which impede progress.

In the early days of the Department of Agriculture there was a Division of Microscopy where, presumably, all of the microscopic work was to be done. The fallacy of this arrangement was, apparently, early recognized, but there grew up later the Bureau of Chemistry, which was to do all of the chemical work for the Department. The difficulties of an organization which confined all of the work in a basic science to one laboratory were not so

evident but when different bureaus, in order to make progress on their problems, found it necessary to establish chemical laboratories this arrangement was abandoned in its turn. Such an arrangement is illogical in a small laboratory, and becomes more and more illogical as the organization grows larger. In solving a biological problem chemistry must be looked on only as a means to the end, a yard stick by which the investigator measures and explains the changes which he observes. The chemical work should be under his control as completely as any of the other tools essential to the solution of his problem.

The organization which usually evolves, if a laboratory grows up naturally, is a force of workers with various qualifications grouped around each problem. A chart showing an organization on this plan usually develops into a fine collection of wheels within wheels. In practice, however, it does not work out as simply as the diagram would indicate. The nature of the plan requires a continual shifting and readjustment of men as the problem develops, or is completed, or abandoned, and new problems are taken up.

It is not always feasible to assign an old and more experienced investigator to work under a younger man who may be responsible for a problem. Some excellent investigators do not have the knack of managing assistants; others do not work well independently, but require close supervision.

The organization around the problem becomes then a question of fitting the arrangement to the workers available, deciding each case on the basis of its own peculiar conditions. It is entirely possible to obtain in one laboratory harmonious and efficient work with the various problems organized in different ways; in one with workers of more or less independence of action responsible to a leader who is in turn responsible to the director; in others with independent workers coöperating and each responsible to the director for his part.

A minor but essential part of the laboratory is that which deals with what may be called the accessory branches, through which the material needs of the organization are provided. These are not coördinate with the investigational staff, but are service units existing only to expedite the work of the investigators.

A general in planning a campaign provides means of transportation, and lines of communication to keep his troops supplied with ammunition. This branch of the service is secondary, but of the most vital importance. If it fails the campaign fails. Its function is to get ammunition to the fighting troops in sufficient quantities and at the time it is needed. The color of the wagons in which it is brought is of little importance. It is of little comfort after the battle is lost for want of shells to know that the records of the ordinance division are complete and accurate.

The clerical force of a research organization performs a similar function. Its function is not to establish channels through which the business of the laboratory must go regardless of consequences, but to provide ways of getting the ammunition to the front when it is needed. The office that has allowed a blind adherence to records and regulation to obscure this end has outlived its usefulness.

The method of administering this service is a difficult one to arrange satisfactorily. Some of the large research institutions relieve the director of all responsibility for financial matters, making the accounting and purchasing division responsible directly to the governing board. Some go to the other extreme, and not only make the clerical force responsible to the director of the laboratory, but place it under the immediate direction of a man of scientific training. The former method would seem to offer large opportunity for the exaggerated idea of the importance and authority which is the great evil of the service branches—the latter should enable the director to make the clerical force what it should be, a servant to the investigational work.

What has been said of the clerical force applies also to the library service, except that with few exceptions the librarians appreciate their relation to the investigational staff and any failure to function properly is usually due to faulty organization rather than to lack of inclination to be of assistance. The contact between the staff and the library must necessarily be a close one. Nothing is more essential to efficiency in research than ready access to the literature. Library administration is not clerical work, but requires special training, a knowledge of

languages, of methods of classification, and above all a wide acquaintance with scientific literature. The library should not be controlled in any way by the clerical force, nor should it be responsible to any particular branch of the laboratory. Its contact with the force should be through the director. It should be so organized that the periodicals are available promptly and the library staff should be in a position to be of real assistance in searching the literature.

A third auxiliary branch which should not be overlooked is the mechanical shop. Even in these days there are scientists whose time is more valuable than that of mechanics and it is not economy to allow work to wait for special apparatus or repairs. In a small laboratory the shop may be only a well equipped work bench; in some lines of investigation the shop is really part of the laboratory, and should be complete and adequately manned. A day rarely passes in a laboratory that some small construction or repair job does not arise. The mechanical branch should be available to take care of this work without unreasonable delay.

Although the results of research are of no immediate value unless an application can be shown, few laboratories outside of the industrial institutions have any organization for the specific purpose of applying new facts and theories to practice. It does not necessarily follow that results are lost because no special provision is made for their utilization, but it is no doubt true that many valuable results are overlooked, and forgotten, because the investigation could not be carried to its logical conclusion.

If Mendel, instead of being an example of the highly individualistic type of investigator, had been a part of a modern research organization, the basic law which he discovered would not have been buried for years, but would have been put into practice at once to the great advancement of plant and animal breeding. Some investigators do not continue their investigations when they reach the applied stage because their tastes lead them to new paths in unexplored fields. In other cases the necessary equipment may not be available, or the branch of human activity to which the results may apply not at hand. The equipment needed may be a factory, a farm, a water system, or a hospital. The

expense and in some investigations the danger to human life may be so great that it becomes advisable to follow the laboratory study with larger but still limited and controlled experiments.

This is generally recognized in industrial laboratories where provision is usually made for carrying an investigation through the laboratory, through a miniature factory test and finally through a full scale factory operation.

In bacteriological work the same course is frequently followed, although the analogy may not be evident at first glance. The soil bacteriologist has his pots and experimental plots, the pathologist his animal rooms and hospital wards.

The efficiency of many other lines of investigation would be greatly increased if definite provisions could be made for carrying investigations to real completion without unnecessary delay. While in some cases it is real economy to encourage an investigator to turn to new problems of a basic nature, leaving the application of his results to others, as a rule the man who develops new principles is the one best fitted by interest and knowledge of the subject to apply them.

Many processes which work well in the laboratory develop defects when tried on a large scale. Many new methods of real value have never gotten beyond this stage, because there was no one with sufficient interest or technical knowledge to adapt the process to the new conditions. Thus there is a great economic loss which can be overcome by proper organization.

And, finally, to complete the work it is necessary to carry the results to that part of the public which is directly concerned. Is the task completed when a technical paper is published in a scientific journal? If its value lies in its use in investigational work it is usually sufficient to make it available to other scientists but if it is something which affects established practices it is only a beginning. The inertia of public opinion cannot be overcome so easily. Even the medical profession, accustomed as it is to rapid advance, cannot always be reached by the printed word alone. New developments in agricultural science permeate very slowly through the journals, the text books, and the teachers to the farms.

The necessity for actual demonstration has become more fully realized, particularly since progress in all fields of science has become so rapid that practice lags behind. The medical profession was convinced of the value of typhoid vaccination by large scale demonstration in the army. The efficiency of administration in introducing new methods in agriculture is now generally recognized and practiced. There is, however, too often a lack of continuity between the investigation and the demonstration. The investigator lacks the facilities for demonstrating his new methods, and the demonstrator is not always sufficiently familiar with the investigational work to select the most valuable results or to avoid mistakes and failures. Real efficiency is obtained when an investigation can go direct from the laboratory to the demonstration stage, with sufficient control retained by the laboratory to insure the use of correct methods.

In the laboratory with which I am connected, if you will pardon the personal allusion, an organization has been perfected which makes this possible. I may cite as an illustration the case of a bacteriological study which proved the relation of certain types of bacteria to the production of the characteristic flavor in Swiss cheese. Through coöperation with a technical man the value of these bacteria in cheese making was determined by miniature factory tests in the laboratory. While this demonstration was very successful, if the work had been stopped at this stage it would still be in the "of scientific interest, but doubtful applicability" class. Everyone who has had experience with pure cultures in the industries knows that there is a wide gulf between the propagation of cultures by a bacteriologist in a laboratory, and by a hand in a factory. Preliminary trials in factories proved that the use of these cheese cultures was no exception. Accordingly, some years were given to operations on a commercial scale in a factory controlled by the laboratory. This not only served to develop and correct some of the difficulties which occur under factory conditions, but also demonstrated the commercial value of the cultures and incidentally trained men for field work.

The actual introduction of these methods into the industry was passed on to another section of the organization, with sufficient control of the methods retained by the laboratory to insure their correct application. The method of introduction consists in demonstration in the factory of the value of the cultures and instruction to the operators in their use.

Thus, we have an example of an organization which provides for basic investigation, for laboratory scale manufacturing tests, for trials under controlled commercial conditions, and finally for carrying the results to the industry by demonstrations and instruction.

If I should attempt to summarize these rambling observations in one sentence I would say that in promoting efficiency in research the essential thing is not the form of the organization but rather the spirit in which it is applied. The organization should be like a roadway over which the investigation passes, not a structure in which it is confined.

Wheeler, from whose address I have already quoted says: "And as the surface of the planet becomes more and more densely covered with its human population, it becomes increasingly necessary to retain portions of it in a wild state, i.e., free from the organizing mania of man, as national and city parks or reservations to which we can escape during our holidays from the administrators, organizers and efficiency experts and everything they stand for, and return to a nature that really understands the business of organization."

Dr. Wheeler has used a more apt illustration perhaps than he realized. The parks to which he would escape to allow his primordial instincts free play are possible only through an efficient organization. The forest through which he roams unhindered has been saved from commercial exploitation by this organization; a fire warden in some isolated lookout station is watching for the first sign of the conflagration which his primitive instinct may have started, and a carefully organized and equipped fire-fighting force is waiting for the telephone message saying that its services are needed at a certain spot; this organization stocks the streams with fish, protects the game, and constructs

roads and trails over which he can flee from the dreaded organizer; in short, it makes it possible for him to escape, as he expresses it, "from the organized routine of our existence."

This conception of a park fits so well with my idea of a properly organized laboratory that I can say with Gratiano, "I thank thee for teaching me that word."

In the ideal research organization the investigator can follow the lure of his elusive problem protected from the distractions of business and finance and propaganda: he can make better progress and enjoy it none the less because the paths have been made smooth and the camp grounds supplied with wood and water. A sense of security should come from the knowledge that a well organized system is protecting him from the consuming conflagration. He may even find it pleasant to exchange notes with the watcher when his path leads him to the look-out station which connects him with the outer world.

REFERENCES

(1) Shull, A. Franklin: Science, 1922, **55**, 497–505.
(2) Wheeler, William Morton: The Organization of Research. Science, 1920, **53**, 53.
(3) Milliken, R. A.: The new opportunity in Science. Science, 1919, **50**, 285–297.

STUDIES ON SALT ACTION

VIII. THE INFLUENCE OF CALCIUM AND SODIUM SALTS AT VARIOUS HYDROGEN ION CONCENTRATIONS UPON THE VIABILITY OF BACTERIUM COLI

C.-E. A. WINSLOW AND I. S. FALK

Department of Public Health, Yale School of Medicine

OBJECTIVES

In an earlier paper (Falk, 1923) our present state of knowledge in regard to the physiological influence of mineral salts upon bacteria has been somewhat comprehensively reviewed. The experiments here reported were designed to determine with some exactness the effect upon bacterial viability of sodium and calcium chlorides, alone and in combination. Our work has extended over a long period, the first preliminary communications (Winslow and Falk, 1918) having been made over four years ago. The early experiments at times yielded conflicting results and it was only by a careful study of the hydrogen ion factor (Falk, 1920) that we succeeded in explaining these inconsistencies along the lines since so well worked out for problems of bacterial growth by Holm and Sherman (1921) and Sherman and Holm (1922). The study presented here deals with the viability of *Bacterium coli* in distilled water, in various concentrations of NaCl, and in various concentrations of $CaCl_2$ with varying hydrogen ion concentrations for each solution; while a following paper (IX) deals with viability in various mixtures of the two salts mentioned above.

TECHNIQUE

All of the experiments here reported were conducted with a single strain of *Bact. coli* which was isolated from a polluted stream near New Haven in the autumn of 1916 and has been

used ever since for various studies of viability conducted in this laboratory.

The bacteria needed for each experiment were cultivated on standard nutrient agar slants, or Petri plates or in Kolle flasks at 37°C. for sixteen to twenty-four hours. The growth from the surface was washed off in water or in the appropriate salt solution, shaken for three to five minutes to break up clumps, filtered through paper or absorbent cotton and then added in 1 cc. portions to the bottles of sterilized water or salt solution in which the viability was to be tested, the solutions having previously been warmed to a temperature of 37°. Quite uniformly the concentration of bacteria at the beginning of each test was 20,000,000 to 40,000,000 per cubic centimeter. Only occasionally was it somewhat lower or higher. Plates were made on agar one minute after seeding to determine the initial number present. The bottles were then replaced in the incubator at 37° and removed for the estimation of the number of surviving organisms at various desired intervals. All counts were made on a standard nutrient agar after incubation for twenty-four hours at 37°C.

The water used in these experiments was carefully distilled from a Barnstead still, or was twice redistilled in glass vessels and again redistilled from glass and into a block tin condenser. Whenever tested it gave a negative test for ammonia with Nessler's reagent. The hydrogen ion concentration of the water used in the earlier experiments was generally between pH 6.4 and 6.8 but was not recorded in regular routine. In certain other tests the initial acidity of the water was higher, usually between pH 5.2 and 5.8.

On the addition of the bacterial cells this reaction would be shifted to a pH of 8.0 or above by the alkaline dissociation products of the suspension. Later there was a tendency for the reaction to return to a zone of hydrogen ion concentration between pH 7.0 and pH 7.4, after an incubation of from one to forty-eight hours. The effect of initial reaction is however often an important one; and in our later work this factor has been carefully controlled.

In those experiments in which we recorded the pH of the solutions, the measurements of acidity were made by the usual

colorimetric procedure, either before or after the addition of the suspension of bacteria according as the pH measurement value "before" or "after" seeding was desired. When the pH of a solution was readjusted, great pains were taken to introduce as few new ions and in as small a concentration as reasonably rapid work permitted. In adjusting the pH of bacterial suspensions in water and in sodium chloride solutions, HCl and NaOH were used. With calcium chloride solutions HCl and $Ca(OH)_2$ were used. The additions of acid or alkali were made by measuring the pH of an aliquot from the test solution, consulting a titration curve of a similar prepared solution to determine the quantity of acid or alkali to be added to give the desired pH and reading the pH of another aliquot after the appropriate addition. Excepting for the water suspensions, the addition of these reagents involved the introduction of no new ions. Because of the comparatively low buffer content of the suspensions in water or in dilute aqueous solutions of these salts, the amounts of acid or alkali added to give the stated pH values were exceedingly minute and the increase in concentration of Na+ and Ca++ ions in the salt solutions incidental to these additions was generally entirely negligible by comparison with the concentration of these ions already present.

Following the work of Cohen (1922) we made some studies of viability in dilute (M/500) buffer solutions. This particular group of studies was carried only far enough to indicate that the buffers, even in such dilution, were producing effects specific to themselves. The results of these experiments are therefore omitted from this paper on the viability of *Bact. coli* in pure water or in solutions of single salts. The study of pH influences upon viability in the absence of buffers is much more difficult and the results from individual experiments are more uncertain than in their presence, but it makes possible observations of certain phenomena which it would be otherwise impossible to observe.

The sodium chloride used in our earlier investigations was purified by treatment with calcium hydroxide (to remove magnesium) and with sodium carbonate (to remove calcium and barium), and was then recrystallized from pure concentrated

hydrochloric acid and from pure water; the calcium chloride was twice recrystallized and the crystals dried in a current of air and in the drying oven. In our later experiments we found that "chemically pure" (analyzed) commercial preparations of these salts were entirely satisfactory when the limiting concentrations of impurities were low.

VIABILITY IN DISTILLED WATER

First of all it was important to determine the behavior of the particular strain of *Bact. coli* used in our experiments when suspended in distilled water free from the presence of notable amounts of mineral salts. An earlier investigation of the viability of this strain by Winslow and Cohen (1918) had shown that it suffered only a slight reduction in numbers when suspended in water for twenty-four hours (82 per cent surviving) but that after this period it gradually died off, only 49 per cent remaining after three days and only 5 per cent after five days.

We ourselves conducted 37 different experiments of this kind during the first years of this study, the results of which are presented in table 1. The pH values at the head of each column indicate the acidity to which the solutions were adjusted immediately after seeding the bottles of water with the bacterial suspensions and at which they were maintained by readjustment of reaction at each observation time if they showed a variation of 0.2 or more from the stated pH. A uniform tendency for the pH to shift towards the zone 7.0 to 7.4 is found here as well as in the experiments with suspensions of *Bact. coli* in sodium and calcium chloride solutions. This point will be treated more fully later. Tests under the column headed "x" were early experiments conducted in solutions, initially alkaline, but in which the pH was neither recorded nor readjusted.

The figures given for the per cent of organisms surviving at each period represent in each case the average of from 2 to 11 tests conducted under the conditions specified, the first seven columns including tests made at known pH values, the last column the earlier tests in which hydrogen-ion concentrations were not recorded. In this and succeeding tables a heavy rule

has been drawn to indicate the range of time and hydrogen-ion concentration most nearly corresponding to a one third destruction of bacteria. There are of course occasional irregularities in the viability curve which make the establishment of this one-third reduction point somewhat conjectural in certain instances as in the columns of table 1 for pH values of 5.0 and 6.0.

It appears from table 1 that at pH 6.0 the reduction in the number of bacteria is very slight, while at pH 5.0 it is somewhat greater; in more acid or more alkaline solutions the viability decreases rapidly. This table, as indicated in its lowest line,

TABLE 1

Viability of Bact. coli in distilled water

HOURS	PER CENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	x
1	87	88	84	92	68	77	79	196
3	39	71	74	66	54	24	52	134
6	4	48	64	30	24	8	12	78
9	1	68	82	7	17	5	12	89
24	0	6	77	2	23	3	10	57
Number of experiments...	2	2	4	2	10	2	4	11

includes the averages of 37 experiments; for the 9-hour period we conducted a more extensive series of tests, this period being chosen because our earlier work had indicated that the physiological influences which we were desirous of measuring were most clearly evident at that time. In table 2 we have collected all of our nine hour data, including those cited in table 1, to give a final table which represents the results of seventy-nine tests.

The curve which expresses the relation between pH and the viability of *Bact. coli* in distilled water is obviously smooth and indicates the existence of a zone of physiological tolerance be-

tween 5.0 and 7.0 which is exceeded more abruptly at the acidic than at the alkaline extreme.

There is some discrepancy between the results of the first 37 tests (table 1) and those of the whole 79 tests (table 2), as would naturally be expected in dealing with a problem of such complexity as the viability of bacteria. Only at pH 7.0 however is this discrepancy serious, the first group of tests showing a 17 per cent survival and the entire series a 54 per cent survival. The results in general confirm the earlier studies of Winslow and Cohen (1918) and indicate that the strain of *Bact. coli* studied persists in distilled water for twenty-four hours with only slightly diminished numbers at a pH of 6.0, but that it shows a sharp reduction in solutions which are more acid than 5.0 or more alkaline than 7.0.

TABLE 2

Viability of Bact. coli in distilled water after nine hours

	pH						
	4.0	5.0	6.0	7.0	7.5	8.0	x
Per cent of bacteria surviving.....	1	82	106	54	35	12	89

It will be noted that the viability for unregulated (and unknown) pH values (in the column headed "x") corresponds approximately to the results obtained at a regulated pH of 6.0, showing little or no decrease in 9 hours. We have reason to believe that the solutions used in these early tests were initially distinctly alkaline (following the addition of bacteria), with a pH in the neighbourhood of 8.0, which would have been expected to show a marked reduction in numbers. The fact that an unregulated alkaline solution gives the same results as a regulated neutral solution, as will be explained in a succeeding paragraph, is due to the buffering properties of the bacterial cells or of their products in solutions whose hydrogen-ion concentrations are not artificially controlled.

Our next problem was to determine the influence exerted upon the viability of our test organism by a salt with a monovalent and one with a bivalent cation. For this purpose we chose the chlorides of sodium and calcium.

THE EFFECT OF SODIUM CHLORIDE IN DIFFERENT CONCENTRATIONS AND AT VARIOUS ACIDITIES

To study the influence of sodium chloride upon the viability of *Bact. coli* we suspended the organisms in salt solutions of varying strengths, from 0.0145 molar (0.1 isotonic) to 1.45 molar (10 isotonic). The technique of manipulation was otherwise the same as that previously described. At the beginning of our work we made up our solutions in fractions and multiples of isotonic strength, following the example of Loeb and other students of the higher organisms. It seems doubtful, however, how far the conception of isotonicity applies to the bacterial cell; and in order to avoid any a priori assumptions we have expressed our data in the present paper in molar terms. For comparability we have, however, kept the same actual concentrations throughout, in spite of the fact that when expressed in molar terms the figures appear as irregular fractions.

Tables 3 to 8 present our findings from 99 experiments in which various concentrations of sodium chloride were studied through twenty-four hour periods under different pH conditions. The figures in table 3 for 0.0145 and 0.0725 molar solutions of sodium chloride show an unquestionably favorable effect on viability. By comparison with the data in tables 1 and 2 which show the mortality in distilled water it is seen that after any incubation period the per cent of bacteria surviving is greater in these salt solutions than in water even at the most favorable pH (6.0). Similarly, table 4 shows that viability is favored in 0.145 molar sodium chloride solution. Even in the four test solutions incubated at pH 5.0 to 6.0 there were still 100 or more per cent of the bacteria alive after 9 or 24 hours as compared with 82 to 77 per cent alive at the favorable pH 6.0 in water. On the other hand a 0.435 molar NaCl solution in one experiment (table 5) conducted at pH 7.3–7.6, was distinctly unfavorable for the survival of *Bact. coli*, and in six experiments in which pH was not regulated a very slight toxicity was manifest, the reduction in numbers, however, being scarcely greater than in distilled water with unregulated reaction. The averages of these 7

TABLE 3

Viability of Bact. coli in sodium chloride solution; 0.1 isotonic (0.0145 M) and 0.5 isotonic (0.0725 M)

HOURS	PER CENT ALIVE	
	0.0145 M—pH not known	0.0725 M—1 experiment pH not known, 1 experiment pH 7.0
1	100	91
3	105	96
6	77	121
9	115	86
24	140	82
Number of experiments.....	7	2

TABLE 4

Viability of Bact. coli in sodium chloride solution; 1.0 isotonic (0.145 M)

HOURS	PER CENT ALIVE			
	ph 5.0—6.0	pH 7.0—7.4	pH not known	All pH values
1	75	90	97*	88*
3	85	91	105	97
6	81	71	101	91
9	101	76	83*	82*
24	114	80	89	84
Number of experiments.....	4	2	10	16

* One unusually high figure omitted from average.

TABLE 5

Viability of Bact. coli in sodium chloride solution; 3 isotonic (0.435 M)

HOURS	PER CENT ALIVE		
	pH 7.3—7.6	pH not known	All pH values
1	94	109	107
3	57	68	66
6	7	71	61
9	0.4	64	55
24	0	63	54
Number of experiments.....	1	6	7

TABLE 6

Viability of Bact. coli in sodium chloride solution; 5 isotonic (0.725 M)

HOURS	PER CENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	Unknown
1	0+	6	82	101	94	98	67	85
3	0+	0.4	78	72	71	38	52	67
6		0.1	54	24	57	26	21	49
9		0.2	43	12	33	11	9	46
24		0.1	13	1	5	0+	0+	30
Number of experiments..	1	4	6	2	10	3	3	18

TABLE 7

Viability of Bact. coli in sodium chloride solution; 6 isotonic (0.870 M)

HOURS	PER CENT ALIVE AT pH				
	6.0	6.5	7.0	7.5	8.0
1	86	86	23	54	55
3	15	35	1	6	6
6	11	6	0.2	0.4	0.2
9	1	1	0+	0+	0+
24	0+	0+	0+	0+	0+
Number of experiments..	A single experiment				

TABLE 8

Viability of Bact. coli in sodium chloride solution; 7 isotonic (1.015 M) and 10 isotonic (1.450 M)

HOURS	PER CENT ALIVE—pH NOT KNOWN	
	1.015 M	1.450 M
1	91	61
3	51	16
6	33	1
9	20	0+
24	8	0+
Number of experiments.....	9	10

experiments indicate that sodium chloride is probably slightly toxic in a 0.435 molar concentration. From the results of 47 experiments with sodium chloride in 0.725 M concentrations summarized in table 6 it appears that a fairly marked toxicity has been attained. When sodium chloride solution exceed 0.725 M concentrations the toxicites of the solutions increase rapidly. In the single experiment conducted with 0.870 molar concentration (table 7) the greatest viability was evidenced at pH 6.0 and 6.5 although the rate of mortality was at all points very great. Concentrations of 1.015 M and 1.450 M (table 8) are uniformly of marked toxicity, killing 80 to 100 per cent of the bacteria in nine hours (table 8).

Throughout all of this work we have found that when measuring viability in non-toxic concentrations of NaCl or in water an occasional test solution will show a decided toxicity not manifested by others of similar strength. Such variations are lost sight of when the data are tabulated in terms of averages as we have done. Lack of space forbids presentation of the individual findings in the hundreds of tests which we have conducted or even a brief discussion of the hypotheses upon which these experimental divagations may be explained. Fantus and Rumry (1920) have demonstrated that neither concentration of inoculum nor presence or absence of clumps is responsible for such variations. With solutions of molar strength the factor of variability appear to be almost completely abolished. In these concentrations NaCl is uniformly and markedly toxic. In general it seems to us that our experiments are sufficiently numerous to warrant the general conclusion that a 0.14 M or less concentration of sodium chloride is favorable to the survival of *Bact. coli;* that a 0.435 M concentration is without marked toxic action upon the bacterium at all pH values studied; that 0.725 M sodium chloride possesses a distinct harmful effect which is least marked in the zone pH = 6.0 to 7.0; and that solutions over a molar strength are powerfully toxic.

Inasmuch as the 99 tests with NaCl which are summarized in tables 3 to 8 were conducted over a period of about three and one-half years, we thought it wise to repeat the series in a single

experiment, in order to determine how accurately we could check the outstanding findings on the relation between concentration and toxicity of this earlier work. We seeded 40 test solutions from a single suspension of *Bact. coli* nineteen hours old in distilled water, kept in an ice bath during the seeding period. The solutions had been sterilized by boiling, instead of in the autoclave, to avoid contamination with ammonia and other volatile substances commonly present in the steam of sterilizers. Before seeding, the solutions had pH values of 8.4 to 8.6, and immediately after seeding with about 35,000,000 *Bact. coli*

TABLE 9

Viability of Bact. coli in various concentrations of sodium chloride

SOLUTION—CONCENTRATION NaCl	SURVIVAL OF BACTERIA AFTER NINE HOURS					
	Experiment 98		All previous experiments		All experiments	
	Per cent alive	Number of tests	Per cent alive	Number of tests	Per cent alive	Number of tests
0	95	5	89	11	91	16
0.0145 M	121	5	115	7	118	12
0.0725 M			86*	2	86*	2
0.145 M	93	5	83	10	86	15
0.435 M	70	5	64	6	67	11
0.725 M	56	5	46	18	48	23
0.870 M	33	5			33	5
1.015 M	18	5	20	9	19	14
1.305 M	6	5			6	5
1.450 M			1	10	1	10

* This figure is introduced to make the tabulation complete. Inasmuch as it is the average of only two tests its validity is perhaps questionable.

per cubic centimeter, of 8.4 to 8.7. For each concentration of sodium chloride there were 5 test solutions. The results of this experiment (Experiment 98) are summarized in table 9 where they are compared with the summarized data of all comparable previous experiments (64 in number) in which viability in sodium chloride was tested and in which there were no adjustments or readjustments of pH.

The unusually close checks obtained renewed our confidence in the accuracy of the results obtained. We may safely conclude that the summarizing column "All experiments" in table 9

describes with reasonable accuracy the viability of *Bact. coli* in solutions of sodium chloride of varying concentration in which no adjustment of pH is made during the course of the test.

When we compare these results, shown in table 9, obtained at unregulated and unknown pH values (but with solutions starting at a pH in the neighborhood of 8.0 or over) with those obtained at a pH value adjusted during the course of the experiment, we find that as with distilled water the unadjusted alkaline solutions give results comparable with those obtained at a regulated pH of about 6.0, (see tables 4, 5, and 6).

EFFECT OF CALCIUM CHLORIDE IN DIFFERENT CONCENTRATIONS AND AT VARIOUS ACIDITIES

Table 10 shows the results obtained with $CaCl_2$ solutions of less than M/10 strength. With the three more dilute solutions (0.00145 M 0.0145 M, 0.029 M) there was the usual variation between parallel bottles always manifest in non-toxic solutions, one bottle showing a decrease, while its companion showed a marked increase. The average results for all the concentrations mentioned, however, show a distinctly higher viability than is manifest in distilled water (96 per cent or more alive after twenty-four hours). These low concentrations, as in the case of NaCl seem to be definitely favorable to viability.

A concentration of 0.072 M $CaCl_2$ on the other hand seems to show toxic action; and with a solution of 0.145 M strength we found an even clearer toxicity, at least *in those solutions in which the pH was not measured or controlled*, (last column of table 11). The bacteria hold their own for the first hour but after that decrease rapidly. After two to three hours only 4 bottles out of 19 in one group, for example, contained over 15 per cent of their original numbers and after twenty-four hours 15 out of 18 bottles showed 1 per cent or less of their original germ content, while the other 3 showed 3 per cent, 10 per cent, and 36 per cent, respectively. For a total of 32 tests at unregulated pH we found 70, 40, 27, 22 and 16 per cent alive after one, three, six, nine and twenty-four hours, respectively. The results obtained with adjusted acidities will be discussed in a succeeding paragraph.

Concentrations of calcium chloride of 0.435 M strength and over showed at all reactions a still more rapid and uniform toxic effect than that indicated for the 0.145 M solution in table 11. Nine tests were made with 0.435 M solutions (table 12). In 6 out of the 9 less than 1 per cent of the bacteria remained after three

TABLE 10

Viability of Bact. coli in calcium chloride solutions; 0.01 isotonic (0.00145 M), 0.1 isotonic (0.0145 M), 0.2 isotonic (0.029 M), 0.5 isotonic (0.072 M)

HOURS	PER CENT ALIVE—pH NOT KNOWN			
	0.00145 M	0.0145 M	0.029 M	0.072 M
1		73	94	88
3	92	84	89	51
6	158	64	105	41
9	166	70	92	45
24	152	96	110	37
Number of experiments.....	2	20	3	5

TABLE 11

Viability of Bact. coli in calcium chloride solutions: 1 isotonic (0.145 M)

HOURS	PER CENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	Unknown
1	42	79	120	127	84	70	86	70
3	1	9	120	108	75	87	99	40
6	0+	2	124	146	66	58	38	27
9	0+	1	78	109	71	61	31	22
24	0	0+	90	61	49	31	12	16
Number of experiments...	1	1	3	2	9	2	2	32

Buffered solutions not included.

hours (the other 3 bottles showing a survival of 19 per cent, 32 per cent and 28 per cent at this period). After six hours these 3 bottles in which the resistance was relatively high showed 2 per cent, 7 per cent and 3 per cent respectively and after nine hours, 0.1 per cent, 1.9 per cent and 0.2 per cent.

Ten tests were made in 0.725 M solutions with the following results. In 5 bottles less than 1 per cent of the bacteria survived after three hours (the per cent surviving in the other 5 bottles being 19 per cent, 3 per cent, 4 per cent, 4 per cent and 9 per cent, respectively). After six hours all had dropped to 1 per cent or less. Five tests in 1.015 M calcium chloride showed less than 1 per cent surviving after three hours in 4 bottles, 1 per cent and 4 per cent in the other 2 cases (table 12).

TABLE 12

Viability of Bact. coli in calcium chloride solutions: 3 isotonic (0.435 M), 5 isotonic (0.725 M), 7 isotonic (1.015 M), 10 isotonic (1.450 M)

HOURS	PER CENT ALIVE—pH NOT KNOWN				
	0.435 M		0.725 M	1.015 M	1.45 M
	pH=7.6				
1	64	37	25	0+	15
3	28	9	4	0+	2
6	3	1	0.4	0	0+
9	0.2	0+	0+	0	0+
24	0	0+	0+	0	0
Number of experiments..	1	9*	10	5	6

* Includes one experiment with pH unknown.

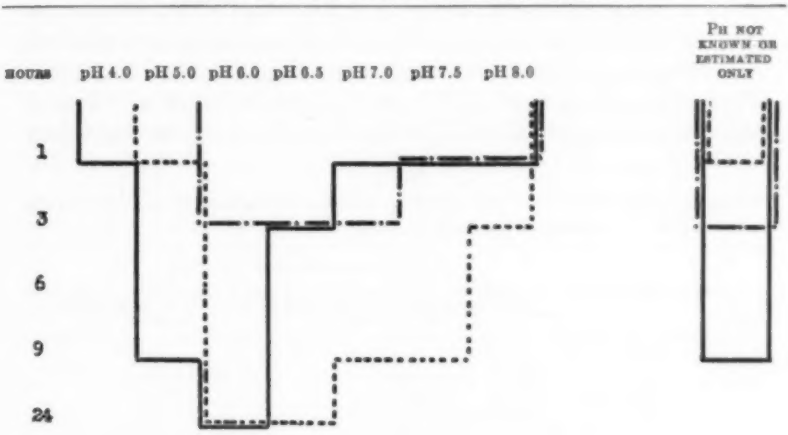
EFFECT OF REACTION IN THE PRESENCE OF SODIUM AND CALCIUM SALTS, RESPECTIVELY

We come now to a consideration of the somewhat puzzling results indicated by table 11 in regard to the influence of the reaction of the calcium chloride solutions upon viability. It has been pointed out above that, in distilled water and in the presence of NaCl, unregulated alkaline solutions (starting in the neighborhood of pH 8.0) show a high degree of viability similar to that obtained at an adjusted reaction of about pH 6.0; and this has been explained as probably due to reversion to a neutral reaction taking place in the presence of the bacterial cells. Yet with $CaCl_2$ the results observed in an unadjusted alkaline solution instead of corresponding with those obtained at a regulated pH of 6.0 show a high toxicity comparable with that characteristic of a regulated pH 8.0.

Tables 13 and 14 show very clearly the apparent conflict in the results observed. These tables include a number of special experiments conducted to cover the nine hour period alone in

TABLE 13

The relation between viability of Bact. coli and pH in distilled water, 5.0 isotonic NaCl (0.725 M) and 1.0 isotonic $CaCl_2$ (0.145 M) solutions



Lines indicate times and reactions at which one-third of the bacteria were dead.
——— Viability in distilled water.
.—. — Viability in 5.0 isotonic NaCl.
..... Viability in 1.0 isotonic $CaCl_2$.

TABLE 14

Effect of pH upon viability of Bact. coli in water, 5.0 isotonic (0.725 M) NaCl and isotonic (0.145 M) $CaCl_2$

SOLUTION	PER CENT OF BACTERIA SURVIVING AFTER NINE HOURS						
	5.0	5.5	6.0	7.0	7.5	8.0	x
Water	82		106	54	35	12	89
5.0 isotonic NaCl	27	20	87	76	8	9	46
1.0 isotonic $CaCl_2$	134		128	106	44	31	22

order to eliminate chance variations by obtaining averages based on a large number of observations. In these tables we have omitted all determinations not based on at least four tests. The results

differ at certain points from those presented in tables 1, 6 and 11 but where this is the case the results in the latter tables were based on 1 or 2 tests only. The bases for tables 13 (136 tests) and 14 (about 250 tests) are sufficiently broad to be reasonably reliable. In water and NaCl solutions the results obtained at an unadjusted alkaline pH correspond approximately to those observed at an adjusted pH of 6.0 or 7.0. In $CaCl_2$ solutions, on the other hand, the results obtained at an unadjusted alkaline pH correspond to those observed at an adjusted pH of about 8.0. Thus at an adjusted pH of 6.0, or at an adjusted pH of 8.0 or at any other adjusted pH, 0.725 M NaCl is slightly more toxic than

TABLE 15

Viability of Bact. coli in 0.145 M calcium chloride with hydrogen ion concentration increased by carbon dioxide from the breath

INCUBATION PERIOD	AVERAGE PER CENT SURVIVING	
	pH decreased by CO_2 from breath (average of 7 tests)	No CO_2 (average of 32 tests—table 12)
hours		
1	139	70
3	114	40
6	75	27
9	67	22
24	52	16
120	3	

0.145 M $CaCl_2$, in an unadjusted alkaline solution on the other hand 0.145 M $CaCl_2$ is distinctly more toxic than 0.725 M NaCl.

This particular concentration of $CaCl_2$ (0.145 M) seems then to display its characteristic toxicity only in an unregulated alkaline solution. The importance of the reaction factor was strikingly called to our attention by the high degree of viability manifest in certain tests in which the suspensions were stirred by blowing into them through the sampling pipettes. Table 15 shows how the carbon dioxide introduced in this way tended to abolish the toxic effect of 0.145 M $CaCl_2$.

An explanation of these phenomena has apparently been found by following out the observations made two years ago (Falk, 1920) as to the power of bacterial suspensions to regulate the pH

of their menstruum and the influence of salts upon that power. These earlier results warranted the assumption that in our experiments in which pH was uncontrolled the initial pH (in each case, an alkaline one) had remained alkaline in the $CaCl_2$ solutions but had shifted towards the neutral point (7.2) in the water and NaCl solutions. We tested this hypothesis in a series of 5 experiments (93 to 98) in which 50 test solutions were studied. We have summarized these results, which have been already reported in preliminary fashion (Winslow and Falk, 1922) in table 16.

It appears that at a pH controlled at about 6.0 neither water nor 0.145 M $CaCl_2$ has toxic action; that in water with an initial pH over 9.0 the reaction falls to about 8.0 in nine hours and no toxic effect is manifest; and, finally, that in a 0.145 M $CaCl_2$ solution of an initial pH over 9.0 the reaction changes but

TABLE 16

Change in pH and viability of Bact. coli in water and 1.0 isotonic (0.145 M) $CaCl_2$

SOLUTIONS	DISTILLED WATER			ISOTONIC $CaCl_2$ (0.145 M)		
	pH initial	After nine hours	Per cent bacteria alive after nine hours	pH initial	After nine hours	Per cent bacteria alive after nine hours
Unadjusted pH	9.2	8.0	91	9.2	8.9	1
Continuously adjusted pH	6.0	6.1	76	6.0	6.5	90

slightly and the bacteria show the high mortality to be expected in such an alkaline solution. It therefore appeared to us that the apparent conflict between the toxicities, absolute and relative, of the test solutions when pH was controlled and when it was uncontrolled, was explicable on the ground that although the unregulated water and sodium solutions on the one hand and calcium solutions on the other may begin an incubation period at the same pH, they do not have the same pH throughout the period. And, hence, the toxicities which are found in such solutions are not comparable with the relative toxicites of solutions kept by careful readjustment at a constant pH.

There remained the question whether the difference in reaction changes was quantitatively adequate to account for the observed differences in viability. The alkalinity of the unadjusted water

solution falls considerably it is true, but does it fall enough to account for the high viability displayed? Thus it appears in table 16 that a pH of 8.0 after nine hours (initial pH = 9.2) is non-toxic while, as previously indicated, a pH *maintained* at 8.0 throughout an experiment is highly toxic. It occurred to us that we might explain this apparent anomaly on the assumption that the regulating action of the bacteria operates in zones immediately surrounding the bacterial cells much more effectively than in the main bulk of the suspending fluid. If therefore the fluid is not stirred frequently or vigorously the bacteria in a solution which they are thus regulating may actually be exposed to a reaction much nearer neutrality than is indicated by a pH reading of the test solution as a whole. In such a case our pH readings do not represent the actual concentration of hydrogen-ions about the cell as they do in a suspension in which reaction is being controlled artificially by repeated shaking, examination of aliquot samples and readjustment of pH.

If this explanation is correct, it should follow that in bacterial suspensions whose pH is not being controlled by readjustments the shift in general pH should be greater in stirred than in unstirred bottles. That is, if in an alkaline suspension the bacteria secrete acidic substances (acidic relative to the pH of the suspensions) the pH of the suspension as a whole will remain comparatively unchanged if for lack of stirring of the solution these acidic substances remain in immediate contact with the bacteria, on the assumption that these acidic substances are secreted only as long as the menstrua are alkaline in the zone about the cell. On the other hand, if the solutions are stirred frequently the neutralizing substances secreted by the cells into the alkaline menstruum will be evenly distributed throughout the solution; the bacteria will be repeatedly exposed to a comparatively alkaline reaction and thus should be induced to secrete more and more of the neutralizing substances.

In order to test this assumption we prepared six bottles of each of the following solutions—water; 0.01, 0.1, 1.0, 3.0, 5.0, 7.0 and 10.0 isotonic NaCl; and 0.01, 0.1, 0.5, 1.0 and 3.0 isotonic $CaCl_2$ (isotonic solution is 0.145 M). All the bottles were set out

on a table where they would not be disturbed and were seeded from a single suspension of *Bact. coli* in distilled water so that each would contain approximately 30,000,000 bacteria per cubic centimeter. The pH of each was read after seeding and adjusted, if necessary, to 8.5 to 8.7. Each group of 6 bottles was then separated by random choice into two groups of 3 bottles each, one group for the "shaken" and the other for the "unshaken" tests. After three, six, nine and twenty-four hours' incubation,

TABLE 17

Effect of mechanical agitation upon change in pH. Suspensions of Bact. coli in water and in NaCl and $CaCl_2$ solutions

SOLUTION—MOLAR CONCENTRATION*	pH CHANGE (INITIAL pH = 8.5—8.7) AFTER INCUBATION FOR							
	Three hours		Six hours		Nine hours		Twenty-four hour	
	Shaken	Not shaken	Shaken	Not shaken	Shaken	Not shaken	Shaken	Not shaken
Water	0.2	0.0	0.9	0.1	1.2	0.2	1.3	0.8
NaCl								
0.00145	1.1	0.7	1.1	1.0	1.2	1.1	1.2	1.2
0.0145	1.1	0.5	1.2	1.2	1.3	1.3	1.3	1.3
0.145	0.4	0.2	1.2	0.9	1.3	1.0	1.2	1.1
0.435	0.3	0.0	1.0	0.1	1.1	0.3	1.2	0.9
0.725	0.3	0.0	1.1	0.1	1.0	0.2	1.1	0.9
1.015	0.7	0.0	0.9	0.1	0.9	0.2	1.2	0.8
1.450	0.0	0.0	0.0	0.0	0.2	0.2	0.9	0.5
$CaCl_2$								
0.00145	0.3	0.0	0.3	0.1	0.4	0.2	1.3	0.5
0.0145	0.4	0.2	0.7	0.2	0.9	0.3	1.2	0.6
0.0725	0.1	0.0	0.6	0.0	0.6	0.1	0.6	0.6
0.145	0.1	0.1	0.2	0.2	0.5	0.2	0.5	0.2
0.435	0.1	0.0	0.2	0.0	0.2	0.0	0.2	0.1

* 1 tonicity = 0.145 M.

pH samples were taken from each bottle. On these occasions, the "shaken" bottles were stirred vigorously before an aliquot was withdrawn. (They were also stirred at one-half hour intervals during the first nine hours between pH readings.) The "unshaken" bottles were never handled and were watched with great care to avoid accidental disturbance. Fine-tipped pipettes were used, and the samples were withdrawn slowly and carefully to avoid stirring and backwash. All of the test solutions were

allowed to become saturated with air before the experiment was begun. For the sake of convenience in manipulation, the work was done at room instead of incubator temperature. The findings are summarized in table 17 where each figure is the average of 3 determinations.

The uniformity of the results in this experiment was very striking. It seems clear that in alkaline solutions the pH tends to shift towards a neutral zone (pH 7.3 to 7.4); that it shifts more rapidly in bacterial suspensions which are being shaken than in those which are not being shaken; that the pH change is greater or more rapid in the less toxic solutions; and that the shift is greater and more rapid in solutions of NaCl than in solutions of $CaCl_2$ of approximately the same toxicity. These results completely confirm our hypotheses and, it appears to us, explain the apparently anomalous results described above. It is evident that relatively slight differences in the general pH of a solution under such conditions must indicate very great differences in the restricted pH of the zones immediately surrounding the bacterial cells.

It appears then that the toxic effect of 0.145 M $CaCl_2$ is an indirect one and is exerted only in an alkaline solution in which the salt interferes with the regulative action exerted by bacterial cells upon the reaction of such a solution.

In his studies on the phenomenon of agglutination Buchanan (1919) has pointed out that under appropriate conditions and in certain concentrations $CaCl_2$ serves as an agglutinating agent with suspensions of meningococci. This suggests that the apparent reduction in numbers of bacteria in $CaCl_2$ solutions may be attributable to agglutination rather than to true toxicity. Although there may be as many viable cells in an agglutinated suspension as in a non-agglutinated one, the number of colonies appearing on an agar-plate will be different in the two cases. Direct microscopic observations of $CaCl_2$ suspensions of *Bact. coli*, however, indicate that with the conditions under which our experiments were conducted agglutination did not ordinarily occur. The rôle of the H-ions present in their influence on agglutinability has not yet, however, been thoroughly studied in this connection.

SUMMARY AND CONCLUSIONS

1. The strain of *Bact. coli* with which we have conducted our experiments maintains itself in distilled water at a favorable pH value without material decrease in numbers for a period of nearly twenty-four hours. Increases are not uncommon during the first few hours. Occasionally however a particular suspension will show a marked decrease due to some cause which we have not yet determined.

2. A reaction of about pH 6.0 is most favorable to the viability of these bacteria in distilled water, the viability decreasing as a solution becomes more acid or more alkaline.

3. A NaCl solution of 0.0145 M strength exerts a distinctly favorable action upon viability. Instead of a slight but definite decrease after twenty-four hours we find that in these highly dilute salt solutions the bacteria maintain themselves in undiminished numbers. The same favorable result is apparent in a $CaCl_2$ solution of 0.00145 M strength.

4. NaCl solutions of 0.725 M strength and over and $CaCl_2$ solutions of 0.435 M strength and over are distinctly toxic at all reactions.

5. Finally a $CaCl_2$ solution of intermediate strength (0.145 M) shows very peculiar and interesting results. At any pH value maintained throughout an experiment by repeated readjustments such a solution is non-toxic. In an unadjusted alkaline solution, however, it displays a definite toxic action which we have demonstrated to be due to the fact that the $CaCl_2$ prevents the bacteria from bringing about a reversion to a more favorable neutral reaction. Our experiments make it clear that in an alkaline solution the bacteria normally alter the medium in the zone immediately adjacent to them in such a way as to decrease its alkalinity to a very marked degree, a process which is inhibited by 0.145 M $CaCl_2$.

REFERENCES

BUCHANAN, R. E. 1919 Agglutination. Jour. Bact., **4**, 73–105.

COHEN, B. 1922 Disinfection studies. The effects of temperature and hydrogen ion concentration upon the viability of *Bact. coli* and *Bact. typhosum* in water. Jour. Bact., **7**, 183–230.

FALK, I. S. 1920 Studies on salt action. III. The effect of hydrogen ion concentration upon salt action. Proc. Soc. Exp. Biol., **17**, 210–211.

FALK, I. S. 1923 Studies on salt action. VII. The rôle of certain ions in bacterial physiology. A review. Abs. Bact., **7**, January, *et. seq.*

FANTUS, B., AND RUMRY, F. 1920 An inquiry into the causes for variation in determinations of disinfecting value. Jour. Inf. Dis., **26**, 351–354.

HOLM, J. E., AND SHERMAN, J. M. 1921 Salt effects in bacterial growth. I. Preliminary paper. Jour. Bact., **6**, 511–519.

SHERMAN, J. M., AND HOLM, G. E. 1922 Salt effects in bacterial growth. II. The growth of *Bact. coli* in relation to H-ion concentration. J. Bact. **7**, 465–470.

WINSLOW, C.-E. A., AND COHEN, B. 1918 Relative viability of *B. coli* and *B. aerogenes* types in water. Jour. Inf. Dis., **23**, 82–89.

WINSLOW, C.-E. A., AND FALK, I. S. 1918 Studies on salt action. I. Effect of calcium and sodium salts upon the viability of the colon bacillus in water. Proc. Soc. Exp. Biol., **15**, 67–69.

WINSLOW, C.-E. A., AND FALK, I. S. 1922 Studies on salt action. IV. The mutual influence of acidity and salt concentration upon bacteria. Proc. Soc. Exp. Biol., **19**, 311–314.

STUDIES ON SALT ACTION

IX. THE ADDITIVE AND ANTAGONISTIC EFFECTS OF SODIUM AND CALCIUM CHLORIDES UPON THE VIABILITY OF BACT. COLI

C.-E. A. WINSLOW AND I. S. FALK

Department of Public Health, Yale School of Medicine

In the immediately preceding paper of this series (Winslow and Falk, 1923) we have presented in some detail the results of our studies upon the influence of two salts with a common anion, one with a typical monovalent and one with a typical divalent cation, upon the viability of *Bact. coli* in water. It was there shown that at any pH value 0.725 M NaCl and 0.435 M $CaCl_2$ solutions are toxic to this organism and that 0.145 $CaCl_2$ is toxic in an alkaline solution (pH 8.0), its toxicity here being due to the fact that $CaCl_2$ under such conditions inhibits the ability of the bacteria to regulate the reaction of the solution.

Over four years ago (Winslow and Falk, 1918) we presented a preliminary report which indicated that in a solution containing sodium and calcium salts in appropriate proportions an antagonistic action may be manifest which tends to protect the bacteria against the toxic action which would be exerted by each salt if present alone. The experiments here reported deal with this question, the technique being the same as that reported in the paper first cited (Winslow and Falk, 1923).

A solution of calcium chloride of 0.145 M strength, the strength at which toxic action first appears in an unadjusted alkaline solution, was chosen for the study of antagonism and in table 1 are presented the results of 21 tests in solutions of calcium chloride of this strength plus various concentrations of sodium chloride. The solution was made up alkaline (about pH 8.0) but was not adjusted thereafter and changes in reaction were not recorded.

In this table we have followed the procedure employed in the previous paper and have drawn a heavy rule to indicate the time after which less than approximately two-thirds of the initial bacterial population are still viable. Table 1 shows that a mixture of NaCl and $CaCl_2$ is moderately toxic when the two are present in equal proportion and each in 0.145 molar ("isotonic") concentration, only 38 per cent of the bacteria surviving six hours or longer. (This strain of *Bact. coli* survives in nearly undiminished numbers—89 per cent—for nine hours' in pure, distilled water.) It shows, further, that the toxic effects of the

TABLE 1

Viability of Bact. coli in solutions containing sodium chloride + calcium chloride

Per cent surviving

	PER CENT ALIVE				
NaCl + $CaCl_2$	1 + 1	2 + 1	3 + 1	4 + 1	5 + 1
Tonicity*	2	3	4	5	6
1	114	65	124	90	165
3	80	52	55	110	89
6	38	27	38	104	44
9	41	28	40	117	30
24 hours	35	28	22	90	0+
Number of experiments	8	3	3	4	3

* 1 tonicity = 0.145 M.

salts are additive, since solutions which contain the same quantity of $CaCl_2$ (0.145 mol per liter) but two and three times as much NaCl (2 and 3 × 0.145 mols per liter) are increasingly toxic. When 4 × 0.145 molar concentration of NaCl is added to 1 × 0.145 molar concentration of $CaCl_2$, however, the toxicity instead of increasing further with the increase in total concentration of the solution diminishes very markedly. In this 5 "isotonic" solution over 100 per cent of the initial number of bacteria are still alive after nine hours as compared with 28 to 41 per cent for a similar incubation period in solutions containing the same

quantity of $CaCl_2$ but lesser quantities of NaCl. Solutions containing 6 × 0.145 mols per liter (5 Na + 1 Ca) become more toxic again but remain less toxic than 3(2 Na + 1 Ca) or 4(3 Na + 1 Ca) times isotonic solutions up to six hours' exposure. In other words, we may state from these experiments, that the toxic influences of NaCl and $CaCl_2$ upon the viability of *Bact. coli* in water are additive up to a certain ratio of Na:Ca after which they become antagonistic.

The additive and antagonistic influences of NaCl and $CaCl_2$ are summarized in somewhat more convenient form in table 2 and in figure 1 where they are presented with the results from

TABLE 2

Viability of Bact. coli in solutions of NaCl and $CaCl_2$, singly and in combinations

TOTAL ISOTONIC CONCENTRATION*	TOTAL MOLAR CONCENTRATION	PER CENT BACTERIA ALIVE AFTER NINE HOURS IN				NUMBER OF EXPERIMENTS AVERAGED		
		Pure NaCl	Pure $CaCl_2$	NaCl + $CaCl_2$	Ratio Na:Ca	NaCl	$CaCl_2$	NaCl + $CaCl_2$
0	0	89	89	89		11	11	11
1	0.145	82	22			16	32	
2	0.290			41	1:1			8
3	0.435	55	0+	28	2:1	7	9	3
4	0.580			40	3:1			3
5	0.725	46	0+	117	4:1	18	10	4
6	0.870	33		30	5:1	5		3

* 1 tonicity = 0.145 M.

comparable experiments with the pure salts used singly. They indicate clearly that in appropriate proportion (4:1), NaCl and $CaCl_2$ are mutually antagonistic to each other with respect to toxicity towards *Bact. coli*. In this proportion and in an absolute concentration in which NaCl by itself is moderately toxic (46 per cent of the bacteria surviving nine hours at 37°C.) and in which $CaCl_2$ is highly toxic (5 iso $CaCl_2$ often produces sterility in nine hours and uniformly reduces the concentration of viable bacteria by more than 99.5 per cent) the mixture of NaCl and $CaCl_2$ permits the bacteria to survive in undiminished numbers for nine to twenty-four hours. Indeed, after nine hours there were still 117 per cent of the bacteria alive as compared to 89 per cent in pure water.

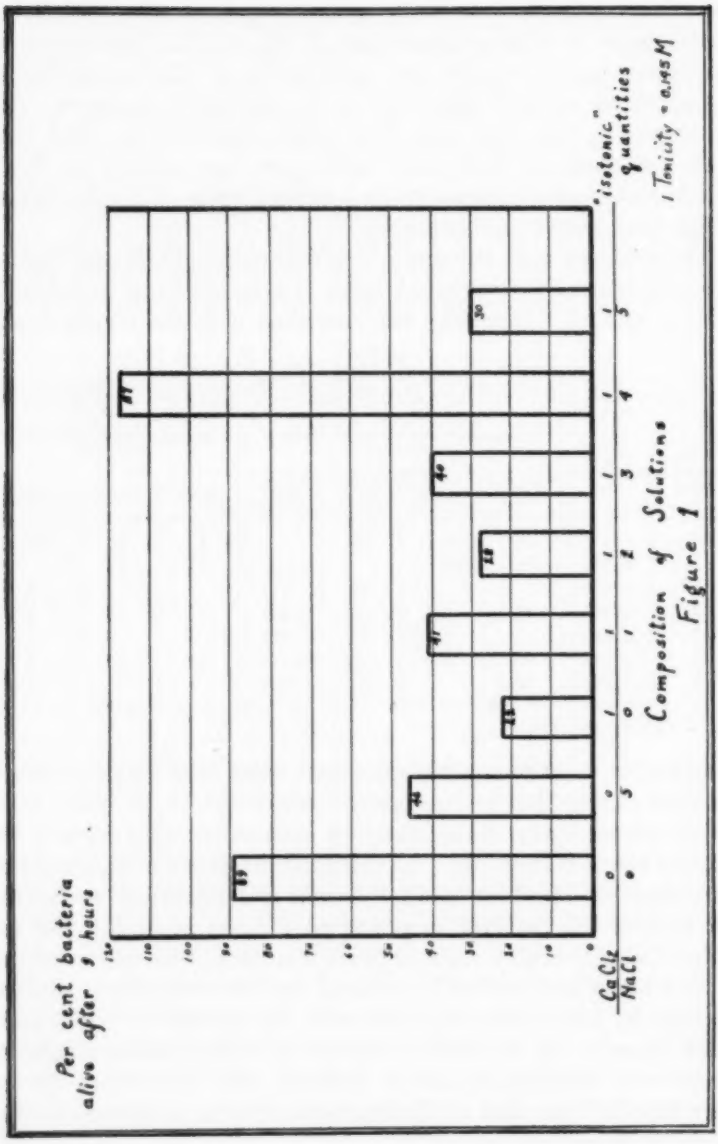

Per cent bacteria alive after 8 hours
100
80
70
60
50
40
30
20
10
0
13
41
11
40
11
30
CaCl2
NaCl
0
3
0
1
2
3
4
5
"isotonic" quantities
Composition of Solutions
1 Tonicity = 0.45M

Figure 1

In the experiments presented thus far no attempt was made to modify or control the pH of the solutions. In a preliminary publication (Falk, 1920) and in the preceding paper in this Journal (Winslow and Falk, 1923) we have indicated certain findings in regard to the influence of hydrogen-ion concentration upon the action of these salts. We have shown that the viability of *Bact. coli* in pure solutions of water, of NaCl and of $CaCl_2$ is greatest at pH 6.0 to 6.5 and diminishes rapidly at a smaller pH and more slowly at a greater pH. The results of 19 experiments on the influence of pH (maintained by repeated readjustment)

TABLE 3

Viability of Bact. coli in solutions containing sodium chloride + calcium chloride

	PER CENT ALIVE						
NaCl + $CaCl_2$	5 + 1 (tonicity = 6)*						
pH	4.0	5.0	6.0	6.5	7.0	7.5	8.0
1	0+	74	88	106	98	92	90
3	0+	86	82	100	84	80	75
6	0+	62	73	92	71	57	54
9	0+	0.2	71	62	50	45	41
24 hours	0+	0+	24	21	13	1	9
Number of experiments	1	1	2	3	6	2	4

* 1 tonicity = 0.145 M.

upon viability in solutions of the two salts, NaCl and $CaCl_2$, are summarized in tabular form in table 3, while in table 4 the nine-hour results are compared with those obtained after the same period in previous experiments by using the same concentration of the salts separately. In the preceding paper of this series we have described in detail the method of adjusting the pH immediately after the bacterial inoculum is added to the test solution and the methods of readjusting the pH if it varied from the stated value by more than 0.2 at stated intervals during the incubation period.

Table 4 and figure 2 bring out the exceedingly interesting fact that at reactions of pH 7.0 and all more acid reactions the effect of NaCl and $CaCl_2$ is additive, the mixture being more toxic than either salt alone. At regulated alkaline reactions on the other hand (comparable to the experiments with unadjusted reaction reported in tables 1 to 3) the two salts antagonize each other, the mixture being less toxic than either salt alone and markedly less toxic than the added toxicities of the two salts used singly. In addition, it is evident from figure 2 that the toxicity of NaCl, at the strength here studied, appears only at pH values below 6.0 or above 7.0; that of $CaCl_2$ only above 7.0. It appears also that NaCl *narrows* and $CaCl_2$ *widens* the zone for hydrogen-ion tolerance of *Bact. coli* and

TABLE 4

Effects on viability of Bact. coli of sodium and calcium chlorides after nine hours' exposure

	PER CENT BACTERIA ALIVE				
pH	5.0	6.0	7.0	7.5	8.0
Water	82	106	54	35	12
0.725 M NaCl	27	87	76	8	9
0.145 M $CaCl_2$	134	128	106	44	31
0.725 M NaCl + 0.145 M $CaCl_2$	0.2	71	50	45	41

that effects of the salts which are clearly evident in more acid solutions tend to disappear (approach the pure water curve) at the same pH (7.0 to 7.5). Holm and Sherman (1921) and Sherman and Holm (1922) have reported that salts which accelerate growth of *Bact. coli* seem to widen and salts which inhibit growth seem to narrow the zone for H-ion tolerance. It is significant to observe that these effects upon growth are confirmed for viability.

In the preceding paper in this Journal we have called attention to the fact that in water and saline suspensions of *Bact. coli*—regardless of the initial pH—the hydrogen-ion concentration tends to shift to the neutral zone 7.0 to 7.4. This observation has also been reported by Eggerth and Bellows (1922). It is

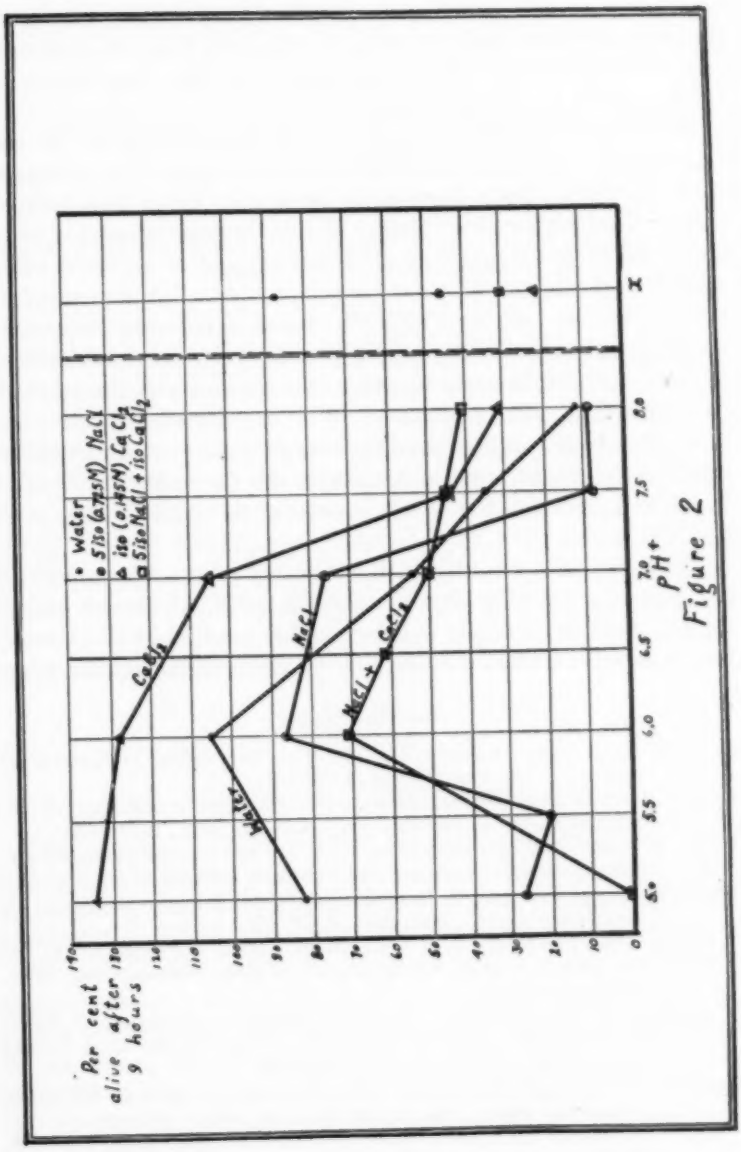

• Water
• 5/16 (0.75M) NaCl
△ 1/8 (0.45M) CaCl2
□ 5/16 NaCl + 1/16 CaCl2
Per cent alive after 9 hours
pH
CaCl2
NaCl
NaCl + CaCl2
Water

Figure 2

interesting to call attention to the fact that all four curves in figure 2 approach uniform values in this pH zone of maximum physiological buffer. The significance of this observation is being studied in our laboratory.

These results, taken together with those reported in the immediately preceding paper seem to us to warrant the conclusion that the toxic effects exerted by salts may be of two distinct sorts. Very high concentrations of salts appear to exert a toxic effect which is apparent at all reactions and is additive when sodium and calcium chlorides are mixed. At a lower concentration (0.145 M) calcium chloride exerts a different influence, manifest only in alkaline solutions and due to an inhibition of the power of the bacteria to reduce the alkalinity of the solution in which they are suspended. It is this latter type of toxic influence which is antagonized by sodium chloride and in alkaline solution the mixture of these salts in the proportion of 5 parts NaCl to 1 part $CaCl_2$ is more favorable to viability than even distilled water.

The mechanism of ionic and molecular effects upon bacteria has been discussed by one of us (Falk, 1923) at greater length than is possible here. It is being further studied in this laboratory and will be treated more fully in later papers in this series.

REFERENCES

Eggerth, A. H., and Bellows, M. 1922 The flocculation of bacteria by proteins. Jour. Gen. Physiol., **4**, 669–680.

Falk, I. S. 1920 Studies on salt action. III. The effect of hydrogen ion concentration upon salt action. Proc. Soc. Exp. Biol., **17**, 210–211.

Falk, I. S. 1923 Studies on salt action. VII. The rôle of certain ions in bacterial physiology. A review. Abs. Bact., **7**, January, *et seq.*

Holm, G. E., and Sherman, J. M. 1921 Salt effects in bacterial growth. I. Preliminary paper. Jour. Bact., **6**, 511–519.

Sherman, J. M., and Holm, G. E. 1922 Salt effects in bacterial growth. II. The growth of *Bact. coli* in relation to H-ion concentration. Jour. Bact., **7**, 465–470.

Winslow, C.-E. A., and Falk, I. S. 1918 Studies on salt action. I. Effect of calcium and sodium salts upon the viability of the colon bacillus in water. Proc. Soc. Exp. Biol., **15**, 67–69.

Winslow, C.-E. A., and Falk, I. S. 1923 Studies on salt action. VIII. The influence of calcium and sodium salts, at various hydrogen ion concentrations upon the viability of *Bacterium coli*. Jour. Bact., **8**, 215-236.

THE FORMOL TITRATION OF BACTERIOLOGICAL MEDIA

J. HOWARD BROWN

From the Department of Animal Pathology of the Rockefeller Institute for Medical Research, Princeton, New Jersey

Received for publication July 2, 1922

The principles involved in the "formol titration" of bacterial cultures or media are the same as those embodied in the methods of Malfatti (1908) and of Henriques and Sörensen (1909) for the titration of urine. They have been applied to the study of bacterial cultures by a number of authors, notably Kendall, Day and Walker (1913), Itano (1916), Berman and Rettger (1918), Foster and Randall (1921), Ayers, Rupp and Mudge (1921), and Kendall (1922). An excess of formalin (approximately 40 per cent formaldehyde) is added to the sample to be titrated. The sample becomes more acid as a result of the following type reactions:

$$R \cdot \underset{\displaystyle COOH}{\overset{\displaystyle |}{CH}} \cdot NH_2 + CH_2O = R \cdot \underset{\displaystyle COOH}{\overset{\displaystyle |}{CH}} \cdot N : CH_2 + H_2O$$

$$4NH_4Cl + 6CH_2O = N_4(CH_2)_6 + 4HCl + 6H_2O$$

The increase in acidity is then titrated with a standard alkali solution. Ammonia, primary amines, and the amino groups of amino-acids, and polypeptids react with formaldehyde. The titration, therefore, represents the sum of these substances.

Sörensen (1907) (1908) has pointed out that the reaction of amino-acids with formaldehyde is a reversible reaction, that an excess of formaldehyde tends to throw the reaction, as expressed in the above equation, from left to right, whereas increasing amounts of water throw the reaction from right to left. Since the methylene derivative of the amino-acid is a stronger acid

than the amino-acid and is therefore more easily titrated, it is obviously desirable to force the reaction from left to right. To do this Sörensen found it necessary to add as much as 10 cc. of formalin to 20 cc. of sample (amino-acid solution), and to titrate with N/5 barium hydroxide or sodium hydroxide. Smaller amounts of formalin were insufficient; larger amounts unnecessary. Workers in bacteriology appear not to have appreciated the importance of this factor. Working with solutions of pure amino-acids Sörensen found it necessary to carry the titration to an end point on the alkaline side of pH 8.2 in order to approximate the theoretical value of some of the amino-acids. He therefore titrated to a deep red color with phenolphthalein as an indicator which he states to have been at about pH 9.0, or to a strong blue color with thymolphthalein which he states to have been at about pH 9.7. Sörensen recognized the presence of carbonates and phosphates as a serious source of error in the formol titrations of amino-acid mixtures containing these substances and to minimize this difficulty titrated with barium hydroxide rather than sodium hydroxide, also adding barium chloride to depress the ionization of the barium carbonate and phosphate formed. The methylene derivatives of some polypeptids and certain of the amino-acids, however, notably tyrosine and phenylalanine, formed insoluble precipitates with barium and for their titration better results were obtained with sodium hydroxide. In the methods of Henriques (1909) and of Henriques and Sörensen (1909) the barium carbonate and phosphate were filtered out, the filtrate neutralized, formalin added, and the acid titrated against sodium or potassium hydroxide.

In developing his method Sörensen made formol titrations of pure solutions of many of the amino-acids and of some peptic, tryptic, and ereptic digests of Witte peptone, casein, and egg albumin. Henriques and Sörensen titrated urines. For the titration of bacterial cultures Itano (1916) followed quite closely the method of Sörensen. Kendall and his associates departed widely from the method of Sörensen in that they used a much larger proportion of water (50 cc.) and a smaller proportion of formalin (5 cc.). Foster and Randall (1921) followed the technic

of Kendall, Day and Walker. Berman and Rettger (1918) departed even more widely from the technic of Sörensen in that they used a total volume of 55 cc. of water and only 2 cc. of formalin. Ayers, Rupp and Mudge (1921) presumably followed Sörensen since they do not mention any modification of the technic. Kendall, Day and Walker and Berman and Rettger departed from Sörensen's method in another respect. They "neutralized" their samples of media to the end point of phenolphthalein, a point which Kendall defines as pH 8.3, before adding the formalin. Sörensen (1908) did not do this. He says, after describing the preparation of the control solution to the "deutliche rote Farbe (zweites Stadium)" of phenolphthalein: "Die zur Untersuchung vorliegenden Lösungen werden bis zu dieser letzten Farbenstärke titriert, indem 20 ccm der Analyse 10 ccm Formolmischung zugesetzt werden, und gleich darauf n/5 Barytlauge bis Rotfärbung, ..."

In the French edition (1907) of his article the meaning is equally clear. He says: "Ensuite on titre jusqu'à la même intensité de coloration les liqueurs à examiner; à cet effet, on ajoute à 20 cc. de la liqueur 10 cc. du mélange de formol et immédiatement après, en agitant bien, la solution de baryte au 1/5, jusqu'à ce que le liquide devienne rouge, ..."

It seems worth while quoting the above passages verbatim because the description of Sörensen's method in certain text books of physiological chemistry would lead one to believe that the solution to be analyzed should be titrated to the color of phenolphthalein produced in the second stage of the control before the formalin is added.

For the titration of solutions of pure amino-acids Sörensen (1907) (1908) apparently carried out no preliminary neutralization or adjustment of hydrogen ion concentration, though when it was necessary to add alkali to get the substance into solution (e.g. tyrosine and aspartic acid) the amount of alkali added was considered in the computation of the formol titration. Later Henriques and Sörensen (1909) (1910) pointed out that the neutral point of the amino-acids is at about pH 6.8 and that this therefore should be the starting point for the titration. They

brought their samples to this reaction by means of a specially prepared litmus paper. Northrop (1921) adopted pH 7.0 with neutral red as an indicator for the starting point of the titration of hydrolyzed gelatin. H. Jessen-Hansen in Abderhalden's Handbuch der biochemischen Arbeitsmethoden, vi, 262, gives a very good discussion of the importance of this factor. Theoretically the isoelectric point should be the point of departure for the titration of amino-acids. Fortunately for our purpose the isoelectric points of the various amino-acids are so close together that their isoelectric zones (Michaelis, 1914, p. 40) overlap and pH 6.8 or 7.0 may be chosen as the ideal starting point for the titration of mixtures of amino-acids and ammonium salts.

The experiments and curves which follow serve to illustrate the importance of choosing the correct hydrogen ion concentrations for the initial and end points of the formol titrations. In bacteriological culture fluids we encounter not only mixtures of amino-acids and ammonium salts but also peptones, polypeptids, fatty acids, carbonates, phosphates, and carbohydrates. The reaction may be acid or alkaline. There are also the disturbing factors of color and turbidity. By known methods of precipitation, filtration, etc. it is possible to eliminate the disturbing factors in such a mixture and to make an accurate formol titration. The present paper is an attempt to analyze the factors involved and to reduce the formol titration of bacteriological media and cultures to its simplest terms.

Phenol red or thymol blue have been used as indicators. The end point is judged by comparison with colorimetric hydrogen ion standards in a comparator block. This not only leads to much greater accuracy in judging the end point but makes it possible to use a color screen of the material being titrated, doing away with the necessity for using artificial colors in a control as was done by Sörensen. Since this work has been in progress Northrop (1921) has also made similar use of the comparator block and colorimetric standards. The titration is carried on in large test tubes containing 1 cc. of sample and 9 cc. of distilled water. The alkali used is N/20 NaOH. For none of the media or cultures titrated to date has it been necessary to use more than 3 cc. of the alkali solution.

As a result of numerous experimental titrations conducted at various hydrogen ion concentrations it is found that optimum results are obtained when 8 cc. of formalin are added to the 10 cc. of diluted sample. Lesser amounts of formalin give lower titrations. It is desirable to use no more formalin than necessary because of the high buffer effect of the formalin itself which results in a poor end point if the titration is carried beyond pH 8.0. More than 8 cc. of formalin have been found unnecessary. The effect of using different amounts of formalin is illustrated in table 1.

In the titrations hereafter reported it is to be understood that 8 cc. of formalin were added to 10 cc. of the diluted sample.

TABLE 1

Effect of various amounts of formalin on the formol titration

SAMPLE (1 cc. + 9 cc. H_2O)	AMOUNT OF FORMALIN			
	1 cc.	5 cc.	8 cc.	10 cc.
Alanine	1.16*	1.94	2.0	2.0
Bouillon	0.9	1.03	1.09	1.09

* The figures represent cc. of N/20 NaOH required for the titration of 1 cc. of sample, the formalin blank[2] having been determined for each amount of formalin and subtracted.

There remain to be determined the limits of hydrogen ion concentration between which titration shall be conducted. If one were to titrate an amino-acid directly against sodium hydroxide, i.e , without the use of formalin, he should start the titration at the isoelectric point of the amino-acid and end it at the point of complete neutralization of the amino-acid. In the case of glycine this would be from pH 6.1 to about pH 11.3. However in the case of glycine and other amino-acids the isoelectric point is in a broad isoelectric zone extending in the case of glycine from about pH 5.0 to pH 7.5 or 8.0. Within this zone the degree of dissociation (a) is very slight and even at pH 8.0 is less than 2 per cent.

$$a = \frac{k_a}{k_a + (H^+)} = \frac{1.8 \times 10^{-10}}{1.8 \times 10^{-10} + 1 \times 10^{-8}} = \frac{1.8}{101.8} \text{ at pH 8.0}$$

If a pure monocarboxylic amino-acid is dissolved in distilled water the solution will be found to have a hydrogen ion concentration at or near the isoelectric point, a fact also noted by Eckweiler, Noyes and Falk (1921). If it is titrated with alkali and the titration curve plotted with amounts of alkali added as abscissae and hydrogen ion exponents as ordinates, as was done in the curves to the left in plate 1, the curve will be seen to drop almost vertically towards the alkaline side as the first drops of alkali are added. This represents a portion of the isoelectric zone. Asparagine (plate 2) behaves as a monocarboxylic amino-acid since in this amide one of the carboxylic groups of aspartic acid is in combination with an amide group. Since by the formol titration it is the carboxyl radical and not the amino radical which is titrated it is important that the monoamino-dicarboxylic acids be converted into mono-carboxylic acids, otherwise their titer will be doubled. This may be accomplished by titrating them with sodium hydroxide to the isoelectric point (or to within the isoelectric zone) of their mono-sodium salts. This zone is represented by the nearly vertical portion of the curves in plate 2. To titrate the nitrogen equivalent of the mono-amino-dicarboxylic acids it is therefore necessary to start the titration within the isoelectric zone of their mono-sodium salts and this zone lies within the same region as that of the mono-carboxylic amino-acids.

Within the isoelectric zone a very minute amount of acid or alkali produces a marked change in hydrogen ion concentration. It makes scarcely any difference in titration at what point we start provided it is within the isoelectric zone. The sodium salts of the amino-acids are not titratable after the addition of formalin since they react with formaldehyde to produce not acids but sodium salts of the methylene derivatives of amino-acids. In a solution of glycine at pH 8.4 ($a = \frac{1.8}{41.8}$) about 4.3 per cent of the glycine is present as the sodium salt. If formalin is added at this point the reaction becomes more acid, or rather less basic, because of the formation of methylene derivatives. However there has already been added sufficient alkali to neutralize 4.3

per cent of the methylenglycine and unless this is taken into account, as was done by Sörensen when he dissolved amino-acids in alkali, the formol titration will fall short by a corresponding amount. Before the formalin is added, mixtures of amino-acids, protein digests, bacterial cultures, etc., should be brought to a reaction within the isoelectric zones of all the amino-acids present if this is possible or unless other considerations make it necessary to compromise in regard to this point. We will call this the initial point of the titration. The end point of the titration should be within what may be called the "zone of neutralization" of the amino-acid if it is titrated directly or within the zone of neutralization of the methylene derivative of the amino-acid if formalin has been added. The term "zone of neutralization" will be used to indicate that portion of the titration curve of an acid and base which extends, on either side of the neutral point, nearly vertically to the abscissa with increasing or decreasing hydrogen ion concentrations plotted as ordinates. The neutral point is the point of inflection of the curve within the zone of neutralization and is reached when just sufficient alkali has been added to convert all of the acid into its alkali salt. The neutral point in the titration of the methylene derivative of an amino-acid is at a higher hydrogen ion concentration than that in the titration of the amino-acid itself and it is upon this fact that the usefulness of the formol titration depends. If formalin is added to solutions of various amino-acids and these mixtures are then titrated with sodium hydroxide, the curves plotted on the right in plates 1 and 2 are obtained. In these curves an almost vertical portion is harder to recognize. This is because of the great excess of formalin in the mixtures titrated. Of the 8 cc. of formalin added to each sample a very small fraction of 1 cc. actually reacts with the amino-acid to form the methylene derivative. The remainder exerts a powerful buffer effect on the alkaline side of pH 8.0. Commercial formalin is acid in reaction. It has been our practice to add to it in preparation for each day's work sufficient normal NaOH to reduce the acidity to about pH 5.0, then to titrate 8 cc. of it plus 10 cc. of distilled water to pH 9.0 or beyond with N/20 NaOH, to plot the titration curve, and

use it for the day's experiments. In plates 1, 2, and 3 points determined on these curves are indicated by crosses. If titration values on the formalin curve are subtracted from values at the same hydrogen ion concentration on the amino-acid plus formalin curve the differences may be plotted as a resultant curve lying between the other two. This resultant curve should be the titration curve of the methylene derivative of the amino-acid. Each one of these resultant curves does reach a nearly vertical position which is the zone of neutralization of the methylene derivative and is the end point of the titration. For each of the substances titrated this zone may be said to begin on the acid side at about the hydrogen ion concentration here listed.

	pH
Glycine	6.8
Phenylalanine	7.6 or 8.0
Asparagine	6.0
Glutamic acid	8.0
Alanine	8.0
Tyrosine	7.6 or 8.0
Aspartic acid	8.0

A similar zone is found in the resultant curve of the formol titration of ammonium salts (plate 3). In these cases the resultant curve represents the titration of hydrochloric, lactic phosphoric, or carbonic acid. The zones may be said to begin at the following hydrogen ion concentrations.

	pH
Ammonium chloride	6.0
Ammonium phosphate	8.0
Ammonium lactate	6.0
Ammonium carbonate	7.0

It will be noted that before the resultant curves reach pH 9.0 their course becomes somewhat irregular or uncertain. This is probably because in the titration of formalin and of formalin plus amino-acids the buffer effect of both substances is so great after they pass beyond pH 8.5 that the end point judged colorimetrically is a very broad and indefinite one. The effect of this is indicated in the cases of glycine, alanine, and asparagine (plates 1 and 2) by shaded regions. The end points of the titrations

might have been taken to lie at any point within these regions. The experimental or technical error is therefore quite large if the titration is carried much beyond pH 8.0 and is in our opinion too large for formol titration by the colorimetric method if carried beyond pH 9.0. The indicators used by us in these regions were phenol red and thymol blue. We found the end point with phenol red at pH 8.0 a very sharp one.[1] At pH 8.0 all of the substances titrated after the addition of formalin had reached the zone of neutralization. This then might be taken as the maximum hydrogen ion concentration at which the formol titration might be completed. An end point of pH 8.2 or 8.4 might be a little better were it not for certain other factors peculiar to the titration of bacteriological media which are discussed below. On the other hand the curves show that pH 8.0 is a little too alkaline to be within the isoelectric zones of some of the amino-acids and ammonium salts. The ideal starting point for formol titration of mixtures of amino-acids would therefore be at a higher hydrogen ion concentration. A hydrogen ion concentration of pH 6.8 as recommended by Henriques and Sörensen (1909) (1910) or of pH 7.0 as employed by Northrop (1921) would serve very well.

However our problem is a special one in that bacteriological media and especially bacterial cultures in such media are more than mere mixtures of amino-acids. They contain among other things volatile and non-volatile fatty acids, phosphates and carbonates, and these substances, especially fatty acids and carbonates together with amino-acids and ammonium salts may change in amount during the growth of the culture. What influence do these substances exert within the range of hydrogen ion concentration chosen for the formol titration? At pH 7.0 the volatile fatty acids are practically neutralized so that they do not enter into a titration with sodium hydrate from pH 7.0 to pH 8.0. The phosphates and carbonates exert large buffer effects between pH 7.0 and pH 8.0, and the amount of alkali

[1] Through the kindness of Dr. van Slyke and Dr. Hastings of the Hospital of the Rockefeller Institute for Medical Research in New York City our color standard at this hydrogen ion concentration was checked electrometrically and found to be correct.

with which they are capable of combining will appear as an error in the formol titration between these limits. If therefore the solution to be titrated is brought to a certain hydrogen ion concentration (e.g., pH 7.0) formalin added and the mixture titrated to a lower hydrogen ion concentration (e.g., pH 8.0 or 8.4) it is absolutely necessary to get rid of the phosphates and carbonates. This may be done by precipitation with barium and filtration.

The following method has given the most accurate results with bacteriological media and with mixtures of amino-acids, phosphates and carbonates.

Method A. Measure out accurately with an Ostwald pipette 2 cc. of culture medium. Add from a burette exactly 2 cc. of N/5 or stronger NaOH. Add a small piece (about 0.2 gram) of barium chloride. Shake to dissolve the barium chloride and allow to stand for a few minutes. Pour onto a small dry paper filter.

With the Ostwald pipette measure out 2 cc. of the filtrate into a large test tube (1 inch in diameter) such as can be used in the comparator block. Caution: Do not blow through the pipette used for measuring the sample since the CO_2 from the breath will cause the fluid to become cloudy.

Add 5 drops of phenol red solution and sufficient N/5 HCl to bring the reaction of the sample near pH 7.0 and then sufficient distilled water to bring the contents of the tube to about 10 cc. which may be judged by a mark on the side of the tube. Bring the reaction of the sample to pH 7.0 with N/20 HCl or NaOH, using the comparator block with 1 cc. of medium plus 9 cc. of water as a color screen behind the colorimetric standard.

Add 4 drops more of the phenol red solution and 8 cc. of formalin.[2] Titrate with N/20 NaOH to pH 8.0 or 8.4. From this result subtract

[2] It is our experience that if formalin is made alkaline it does not "keep" well, *i.e.*, the hydrogen ion concentration increases slowly but appreciably within two hours. If, however, it is made slightly acid it does not change appreciably during the working day. Instead of adjusting the reaction of the formalin to pH 8.0 or 8.4 before adding it to the sample of medium we prefer to prepare it as follows: To a sufficient quantity for the days' work is added normal NaOH (usually less than 1 cc. per 100 cc. of formalin) until the reaction is between pH 5.0 and pH 7.0. The formalin blank consists of 8 cc. of this formalin plus 10 cc. of distilled water titrated against N/20 NaOH to pH 8.0 or 8.4 (the end point chosen for the formol titrations).

the titration of the formalin blank.[2] The remainder multiplied by 5 is the formol titration expressed in terms of per cent normal, *i.e.*, the percentage normality of substances reacting with formaldehyde to produce titratable acids.

In the formol titration of bouillon we have obtained exactly the same values when titrating to pH 8.0 as at pH 8.4 whereas the colorimetric end point at pH 8.0 is somewhat sharper than at pH 8.4 because of the greater buffer effect of the formalin at pH 8.4.

A briefer method than the above, one which requires less material and which gives almost identical results even in the presence of large amounts of carbonates and phosphates in bouillon, is as follows:

Method B. With an Ostwald pipette measure out 1 cc. of the medium or culture into each of two large test-tubes (1 inch in diameter) such as can be used in the comparator block. To each tube add 9 cc. of ammonia-free distilled water or better sufficient water to bring the contents of both tubes to the same level. One of the tubes serves as a color screen in the comparator block. To the other tube, hereafter referred to as the sample, add 5 drops of phenol red (the same proportion of indicator as is contained in the colorimetric hydrogen ion standards).

Bring the reaction of the sample to pH 8.0 by the cautious addition of of N/20 NaOH or HCl[3] as needed. A few drops are usually sufficient.

To the sample add 4 drops more of phenol red and then to both the sample and the color screen tubes add 8 cc. of formalin.[2] Twirl the sample tube just sufficiently to mix the formalin with the sample and as quickly as possible but with as little agitation as possible, add from a burette[3] N/20 NaOH until the pink color of the indicator is visible, then more deliberately titrate to pH 8.0. After subtracting the titration of the formalin blank the result multiplied by 5 is the formol titration expressed in terms of per cent normal. By multiplying the latter result by 14 the result may be expressed as milligrams of nitrogen per 100 cc. of medium.

[3] The burette used should be finely graduated so that it can be read in hundredths of a cubic centimeter. The N/20 NaOH and HCl used should contain phenol red in the same concentration as is present in the sample and in the colorimetric standards, a suggestion adopted from Hurowitz, Meyer, and Ostenberg (1915).

The method above described resembles that of Kendall, Day and Walker (1913) and that of Berman and Rettger (1918) in that the sample is brought to a certain hydrogen ion concentration before formalin is added and titrated back to the same hydrogen ion concentration after the addition of the formalin, but it differs from them in other respects. Kendall, Day and Walker allowed the sample of medium to stand thirty minutes after the addition of formalin before proceeding with the titration. We have found this not only unnecessary but distinctly bad because of the loss of CO_2 into the atmosphere. The CO_2 may be removed before the formalin is added in which case any ammonium carbonate which was present is transformed into other ammonium salts and there will be no further loss of CO_2 after the formalin is added, but the escape of CO_2 after the formalin is added means a decreasing titratable acidity and hence lowers the formol titration. The CO_2 must either be removed before adding the formalin or must be kept in solution until the titration is finished. If in the presence of carbonates it is desired to allow the mixture of formalin and sample to stand for a few minutes it should first be brought to an alkaline reaction. The effect of the escape of CO_2 into the air is illustrated by the formol titration curve of ammonium carbonate in plate 3. This titration was carried out with four different indicators in four different samples of ammonium carbonate solution. Formalin was added to the first sample (methyl red) and it was titrated deliberately from pH 5.2 to 5.6. Formalin was added to a second sample (brom cresol purple) and it was titrated from pH 6.0 to 6.8. Similarly a third sample (phenol red) was titrated from pH 7.0 to 8.4. The fourth sample (thymol blue) was titrated from pH 8.8 to 9.0. It will be noted that as the titration of each sample progressed the curves ending on the acid side of pH 7.0 fell short, so as to produce breaks or steps in the entire curve at A and B, a condition which did not occur in the absence of carbonates, as is illustrated by the curves of the other substances similarly titrated. We attribute these breaks to loss of CO_2. This is further illustrated in table 2.

TABLE 2

Illustrating the effect of loss of CO_2 during the formal titration

SAMPLE	PROCEDURE	RESULT
Glycine	Titrated deliberately	2.53*
Glycine + 1 per cent $NaHCO_3$	Titrated several minutes after addition of formalin	2.34
Glycine + 1 per cent $NaHCO_3$	Titrated quickly after addition of formalin	2.52
Glycine + 1 per cent $NaHCO_3$	Titrated under oil	2.55
Glycine + 1 per cent NaH_2PO_4	Titrated deliberately	2.54

* In this and the succeeding tables the results are expressed as cubic centimeters of N/20 NaOH required for the titration of 1 cc. of sample, the formalin blank[3] having been subtracted.

TABLE 3

Formol titrations in the presence of carbonates and phosphates

SAMPLE	INITIAL POINT OF TITRATION	END POINT OF TITRATION	RESULT
	pH	*pH*	
Bouillon only (containing only the phosphates and carbonates which are native to meat infusion bouillon)	7.0	8.0	1.35
	7.0	8.4	1.35
	8.0	8.0	1.07
	8.4	8.4	0.96
Bouillon + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (titrated quickly)	7.0	8.0	1.9
	8.0	8.0	1.06
Bouillon + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (carbonates and phosphates precipitated by $BaCl_2$ and filtered out)	7.0	8.0	1.13
Glycine only	7.0	8.0	1.24
	8.0	8.0	1.22
Glycine + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (titrated quickly)	8.0	8.0	1.22
Glycine + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (carbonates and phosphates precipitated by $BaCl_2$ and filtered out)	7.0	8.0	1.26

By bringing the reaction back to alkaline as quickly as possible after the addition of formalin the loss of CO_2 into the air is minimized. By adjusting the reaction of the sample to pH 8.0 and carrying the final titration to the same point the buffer effect of phosphates, carbonates, or other buffer substances in the medium is eliminated. That by these means the necessity for removing the phosphates and carbonates by precipitation is obviated is illustrated in tables 2 and 3. Other means have been tried, such as preliminary acidification and boiling or aeration to get rid of the CO_2, or titration under a layer of oil, but the technic described above is almost if not quite as efficient and much simpler.

From a study of the titration curves of pure amino-acids and ammonium salts already described it was concluded that optimum results were obtained by adjusting the reaction of the sample to pH 6.8 or 7.0 then adding the formalin and titrating to pH 8.0 or 8.4. However, with bouillon, and particularly if phosphates or carbonates have been added, titration between these limits of hydrogen ion concentration is impossible as is also illustrated in table 3. To eliminate the buffer effect of these substances the formol titration must start and end at the same hydrogen ion concentration. It is therefore imperative to determine what this hydrogen ion concentration should be. For each of the pure substances titrated formol titrations were made between such limits as pH 5.6 to 5.6, pH 6.8 to 6.8, pH 7.0 to 7.0, pH 7.6 to 7.6, pH 8.0 to 8.0, pH 8.4 to 8.4, and pH 9.0 to 9.0. These titrations are indicated by small triangles in plates 1, 2 and 3.[4] The results are in strict agreement with what might be deduced from the character of the other curves plotted. If the isoelectric zone of the amino-acid overlaps a portion of the zone of neutralization of the methylene derivative (resultant curve) (i.e., if the two curves fall nearly vertically at

[4] For all of the solutions, the titrations of which are plotted in plates 1, 2 and 3, total nitrogen or ammonia determinations were made. On the assumption that these determinations represent 100 per cent of the substance in solution the curves are so adjusted that a perfect formol titration (*i.e.*, titration of 100 per cent of the substance) should require 2 cc. of N/20NaOH. A solution of this concentration would be one-tenth normal.

the same hydrogen ion concentration) the formol titration gives practically perfect results when carried out between limits of hydrogen ion concentration within the overlapping portion of these zones. For example the isoelectric zone of glycine (plate 1) and the zone of neutralization of its methylene derivative (resultant curve) overlap from about pH 6.8 to 8.0 and practically 100 per cent of the glycine may be determined by formol titration from pH 6.8 to 6.8 or from pH 8.0 to 8.0. However on either side of these extremes, as shown by the triangles at pH 5.6, 8.4 and 9.4, lower values are obtained. In the case of alanine there is only slight overlapping of the zones at pH 8.0 to perhaps 8.4. In the case of asparagine, ammonium chloride and ammonium lactate there is overlapping from about pH 6.0 to 7.0. In the case of the other amino-acids titrated there is no actual overlapping of the zones, consequently with these substances all formol titrations which begin and end at the same hydrogen ion concentration are too low, and if we are to use the method under consideration the hydrogen ion concentration at which a maximum titration is obtained must be found. Maximum titration values for certain amino-acids were obtained at the following hydrogen ion concentrations; phenylalanine, pH 7.6; tyrosine, pH 8.0; aspartic acid, pH 8.4; glutamic acid, pH 8.0 to 8.4. We did not have the difficulty experienced by Sörensen (1907) (1908) of obtaining results that were too high in the formol titration of tyrosine. With ammonium phosphate and ammonium carbonate another phenomenon is encountered; the line of the formol titrations starting and ending at the same hydrogen ion concentration crosses the titration curves of phosphoric and carbonic acids (resultant curves). The formol titration of ammonium phosphate at points to the acid side of pH 7.8 leads to results which are too high while titration at points to the alkaline side of pH 8.0 gives low results. Perfect results are obtained at pH 7.8 or 8.0. Formol titration of ammonium carbonate did not yield 100 per cent of the ammonia value probably because of the escape of some of the CO_2 during titration, but maximum results (at least 90 per cent of the theoretical) were obtained at pH 6.0, 7.0, or 8.0.

If it is desired to perform a formol titration of a mixture of amino-acids and ammonium salts from a selected hydrogen ion concentration back to the same hydrogen ion concentration, as here proposed, the optimum hydrogen ion concentration will evidently depend upon the relative proportions of the various amino-acids and ammonium salts present. This optimum hydrogen ion concentration has been determined empirically for solutions of "aminoids," peptone and standard meat infusion bouillon (see table 4).

TABLE 4

Formol titrations of bacteriological media at various hydrogen ion concentrations

MEDIUM	THE INITIAL AND END POINTS OF TITRATION				
	pH 7.2–7.2	pH 7.6–7.6	pH 8.0–8.0	pH 8.4–8.4	pH 9.0–9.0
1 per cent beef aminoids*	0.86	0.9	0.95	0.79	0.64
1 per cent casein aminoids*	0.68	0.71	0.74	0.65	0.36
1 per cent Fairchild peptone	0.68	0.67	0.68	0.52	0.39
	(pH 7.0–7.0)				
Veal bouillon 1502	1.04	1.12	1.11	1.04	
Veal bouillon 1502 + 1 per cent NaH_2PO_4	1.35	1.23	1.10	1.04	
Veal bouillon 1502 + 1 per cent $NaHCO_3$	0.81	1.04	1.07	0.99	

* Arlington Chemical Co. product.

The "aminoids" gave maximum formol titrations at pH 8.0. Fairchild peptone gave maximum results between pH 7.2 and pH 8.0. The veal infusion bouillon gave maximum titrations at pH 7.6 and pH 8.0. In the case of other samples of bouillon we have found the maximum more often at pH 8.0 than at pH 7.6. When $NaHCO_3$ or NaH_2PO_4 was added to the bouillon practically the same result was obtained at pH 8.0, but in the phosphate bouillon higher titrations were obtained at pH 7.6 and at pH 7.0. This phenomenon is related to a similar peculiarity of the ammonium phosphate curve (plate 3) already commented upon. This fact and the comparison with the titrations of the plain bouillon and other substances serve to indicate that the formol titration of bouillon from pH 8.0 to 8.0 gives the most

nearly correct value, a value very close to that which may be obtained after removal of the phosphates and carbonates. In the case of pure amino-acids and ammonium salts the formol titration from pH 8.0 to 8.0 yields from 90 to 100 per cent of the theoretical value, depending upon the individual substance titrated.[5]

It will be of interest to know how the results so obtained compare with those obtained by the methods of Kendall, Day and Walker (1913), Foster and Randall (1921), Kendall (1922),

TABLE 5

Comparison of the results of formol titration by the methods of Kendall, Berman and Rettger, and the method here described

SAMPLE	METHODS OF		
	Kendall	Berman and Rettger	The author
Glycine	2.27*		2.58
Asparagine	0.86		1.11
Veal bouillon	0.86	0.65	1.07

* For purpose of comparison all the results are reduced to the same terms. The figures represent cc. of N/20 NaOH required for the titration of each 1 cc. of sample, the formalin blank[3] having been subtracted in the author's method, the formalin having been "neutralized" to phenolphthalein in the methods of Kendall and of Berman and Rettger.

and Berman and Rettger (1918). The results recorded in table 5 serve to show that the results of Kendall's method are probably at least 20 per cent too low and those of Berman and Rettger even lower. Assuming that the ammonia determinations in the work of these authors were correct it will be seen that when the ammonia nitrogen was subtracted from the formol nitrogen the resulting amino nitrogen value probably involved an error even greater than that indicated by the formol titration alone. Three factors must have conspired to produce these

[5] Eckweiler, Noyes and Falk (1921) have published a titration curve of glycylglycine which is said to be similar to that of other simple dipeptides. It is to be noted that according to this curve the formol titration of these substances from pH 8.0 to 8.0 would introduce a larger error than that found in the titration of simple amino-acids. However, in the case of standard meat infusion bouillon at least, the sum of errors is not a large one.

errors: (1) the use of too much water and too little formalin, (2) the titration from pH 8.3 or 8.4 (the end point of phenolphthalein) back to pH 8.3 or 8.4, and (3) the loss of CO_2 from the sample after formalin had been added.

In criticism of the method of Sörensen it was claimed by de Jager (1909) that a mixture of glycine and ammonium salt gave a formol titration which was lower than the sum of the formol titrations of the glycine and ammonium salt made separately. Henriques and Sörensen (1910) found this of little consequence

TABLE 6

Formol titration of mixtures of an amino-acid and an ammonium salt

SAMPLE	FORMOL TITRATION (pH 8.0 TO 8.0)
1 cc. glycine solution	2.53
1 cc. ammonium lactate solution	1.37
Sum	3.90
Mixture of 1 cc. glycine solution and 1 cc. ammonium lactate solution	3.91

TABLE 7

Formol titration of an amino-acid added to bouillon

SAMPLE	FORMOL TITRATION (pH 8.0 TO 8.0)
1 cc. glycine solution	1.17
1 cc. bouillon	1.09
Sum	2.26
Mixture of 1 cc. glycine solution and 1 cc. bouillon	2.26

in the titration of urines. In table 6 it is shown that this is not a disturbing factor in the method here described.

In the growth of bacterial cultures amino-acids are often liberated from protein substances in the medium. It is therefore of direct interest to know whether the amount of such acid liberated may be actually measured by the formol titration. The formol titration of bouillon before and after the addition of a titrated amount of glycine indicates that it can be so measured (see table 7).

SUMMARY

The influence of certain factors involved in the formol titration of amino-acids and ammonium salts is illustrated. Their titration curves are plotted.

Serious errors in current methods of performing the formol titration of bacteriological media are pointed out.

It is shown that the formol titration of bacteriological media presents a special problem. Simple methods of performing this titration are described, methods requiring very small samples of media. Method A is a modification of the technique of Henriques and Sörensen for the formol titration of urine and involves the removal of carbonates and phosphates by precipitation with barium. Method B is almost as accurate as A, yielding results which probably represent 94 per cent of the amino-acids and ammonia present, and does not require the removal of carbonates and phosphates.

It is a pleasure to express my gratitude to Dr. Paul E. Howe of this Department of the Rockefeller Institute for many valuable suggestions and criticisms during the course of the work, and also to Dr. D. D. van Slyke and Dr. J. H. Northrop of the Rockefeller Institute in New York City for criticism of the manuscript.

REFERENCES

AYERS, S. H., RUPP, P., AND MUDGE, C. S. 1921 The production of ammonia and carbon dioxide by streptococci, Jour. Inf. Dis., **29**, 235.

BERMAN, N., AND RETTGER, L. F. 1918 Bacterial nutrition: Further studies on the utilization of protein and non-protein nitrogen, Jour. Bact., **3**, 367.

ECKWEILER, H., NOYES, H. M., AND FALK, K. G. 1921 The amphoteric properties of some amino-acids and peptides, Jour. Gen. Physiol., **3**, 291.

FOSTER, L. F., AND RANDALL, S. B. 1921 A study of the variations in hydrogen ion concentration of broth media, Jour. Bact., **6**, 143.

HENRIQUES, V. 1909 Über quantitative Bestimmung der Aminosäuren im Harne. Z. f. physiol. chem., **60**, 1.

HENRIQUES, V., AND SÖRENSEN, S. P. L. 1909 Ueber die quantitative Bestimmung der Aminosäuren, Polypeptide und der Hippursäure im Harne durch Formoltitration, I. Z. f. physiol. Chem., **63**, 27.

HENRIQUES, V., AND SÖRENSEN, S. P. L. 1910 Ueber die quantitative Bestimmung der Aminosäures, Polypeptide und der Hippursäure im Harne durch Formoltitration, III. Z. f. physiol. Chem., **64**, 120.

HUROWITZ, S. H., MEYER, K. F., AND OSTENBERG, Z. 1915 On a colorimetric method of adjusting bacteriological culture media to any optimum hydrogen ion concentration. Proc. Soc. Exp. Biol. and Med., **13**, 24.

ITANO, ARAO 1916 The relation of hydrogen ion concentration of media to the proteolytic activity of *Bacillus subtilis*, Mass. Ag. Exp. Station Bulletin, No. 167.

DE JAGER, L. 1909 Beiträge zur Harnchemie. Z. f. physiol. Chem., **62**, 333.

KENDALL, A. I., DAY, A. A., AND WALKER, A. W. 1913 Studies in bacterial metabolism, XIII–XXX, Jour. Am. Chem. Soc., **35**, 1201.

KENDALL, A. I. 1922 The significance and quantitative measurement of the nitrogen metabolism of bacteria. Jour. Inf. Dis., **30**, 211.

MALFATTI, H. 1908 Eine klinische Methode zur Bestimmung des Ammoniaks im Harne, Z. f. anal. Chem., **47**, 273.

MICHAELIS, L. 1914 Die Wasserstoffionenkonzentration. Berlin.

NORTHROP, J. H. 1921 The rôle of the activity coefficient of the hydrogen ion in the hydrolysis of gelatin, Jour. Gen. Physiol., **3**, 715.

SÖRENSEN, S. P. L. 1907 Études enzymatiques. Comptes-rendus des Travaux du Lab. de Carlsberg, **7**, 1.

SÖRENSEN, S. P. L. 1908 Enzymstudien, Biochem. Zeitschr., **7**, 45.

EXPLANATION OF PLATES

1. —⊙—⊙— titration curve of 1 cc. of sample plus 9 cc. water.
2. —×—×— titration curve of 8 cc. formalin plus 10 cc. water.
3. —⊕—⊕— titration curve of 1 cc. sample plus 9 cc. water and 8 cc. formalin.
4. —•—•— resultant curve obtained by subtracting values on curve 2 from those on curve 3.
5. △ formol titrations.

Note: The substance titrated, i.e., the sample, is indicated by the name printed beneath each set of curves.

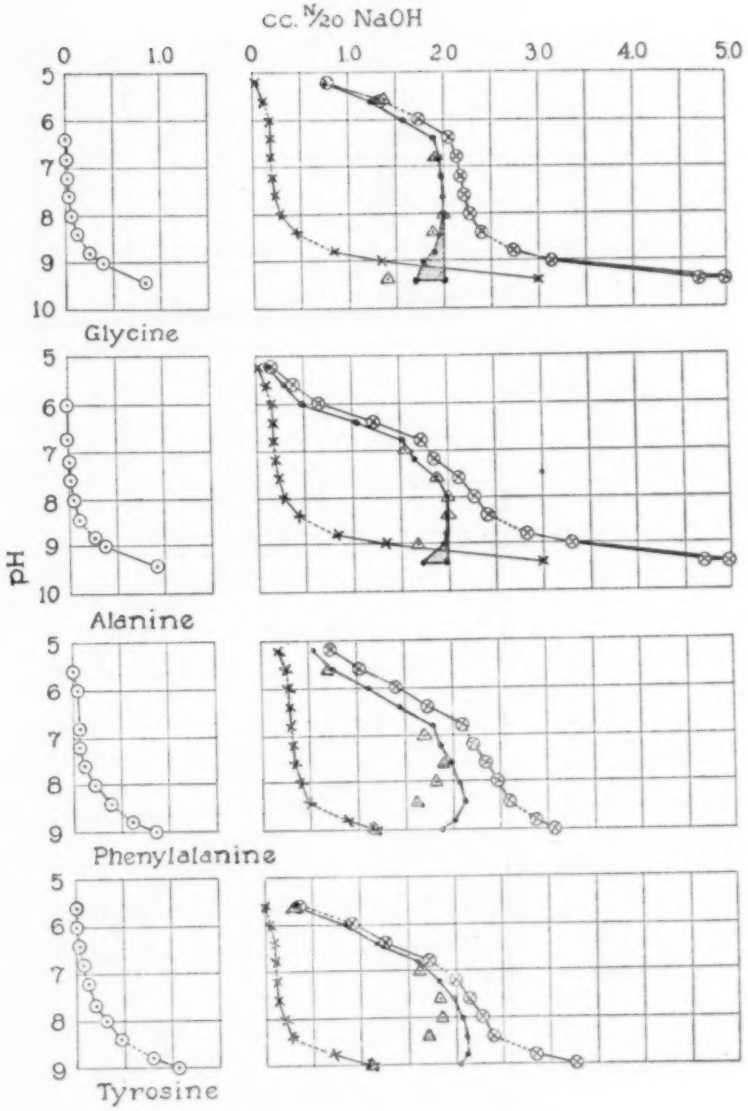

cc. N/20 NaOH
0
1.0
0
1.0
2.0
3.0
4.0
5.0
pH
5
6
7
8
9
10
Glycine
Alanine
Phenylalanine
Tyrosine

PLATE 1

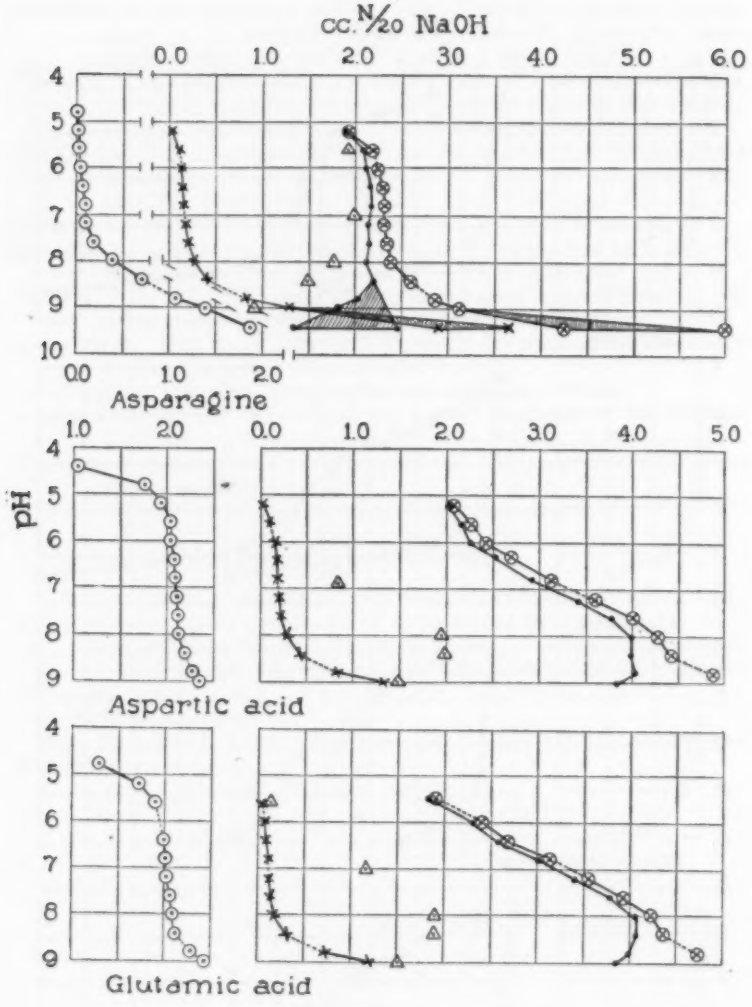

cc. N/20 NaOH
0.0
1.0
2.0
3.0
4.0
5.0
6.0
pH
4
5
6
7
8
9
10
Asparagine
Aspartic acid
Glutamic acid

PLATE 2

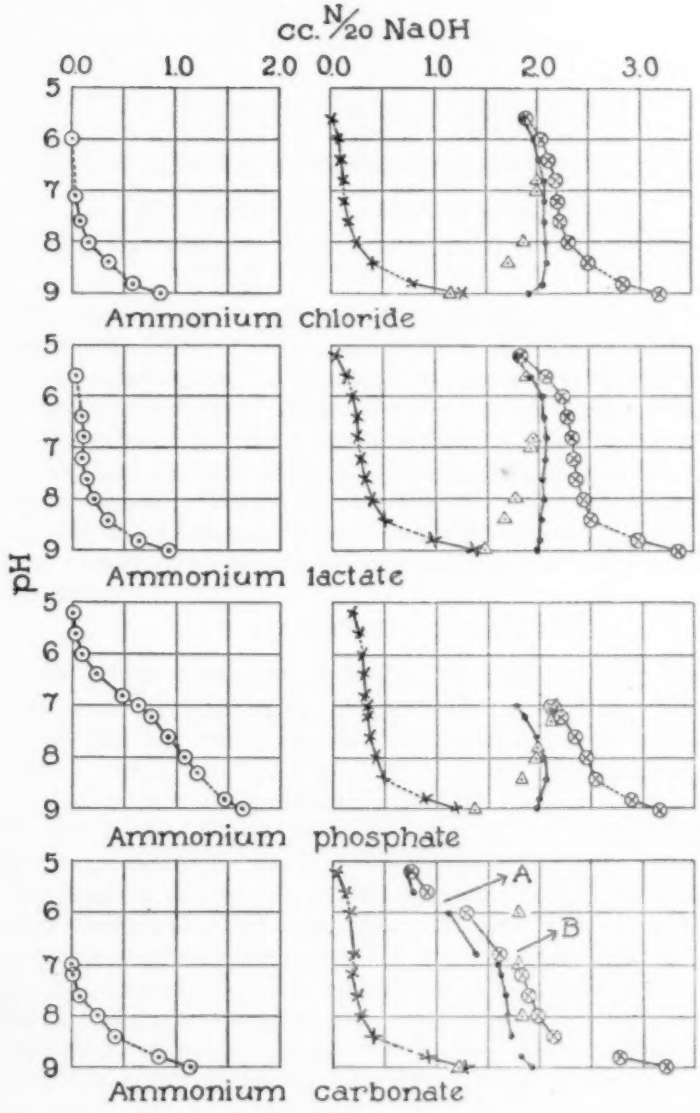

cc. N/20 NaOH
0.0
1.0
2.0
0.0
1.0
2.0
3.0
pH
5
6
7
8
9
Ammonium chloride
Ammonium lactate
Ammonium phosphate
Ammonium carbonate
A
B

PLATE 3

CLOSTRIDIUM BOTULINUM

IV. RESISTANCE OF SPORES TO MOIST HEAT

FRED W. TANNER AND FORREST D. McCREA

From the Department of Bacteriology, University of Illinois, Urbana, Illinois

Received for publication July 5, 1922

The several recent outbreaks of botulism, which have been traced to foods preserved by some method of heating, have focused critical attention on the thermal resistance of the spores of *Clostridium botulinum*. While several papers have been published on this subject, its importance justifies repetition of the work in order that the conclusions based on the data, may be as accurate as possible. The present investigation was initiated before some of the other papers were published and was continued even though the experiments repeated some of those already reported.

HISTORICAL

Two papers have been published by Weiss (1921a, 1921b) which bear directly on the subject under discussion. He reported that the free spores of *Clostridium botulinum* were destroyed within five hours at 100°C., within forty minutes at 105°C., and within six minutes at 120°C. Weiss believed that the spores were injured before death and that the death process was a gradual one. Such an opinion is in keeping with our knowledge of disinfection by moist heat. Young spores were more resistant than old ones. Those one month old were found to be many times more resistant than older ones. The hydrogen ion concentration was found to have the usual effect. In the second paper Weiss reported some data on the thermal resistance of spores in canned food liquors. The resistance, as would be expected, varied with the reaction of the food. In gooseberry juice the spores were killed in 30 minutes while about

180 minutes were required for killing the spores in lima bean juice. Burke (1919 a) reported that the spores could survive boiling for three hours or more. In another paper Burke (1919 b) reported that the spores grown in brain medium were more resistant than those grown in broth. Fifteen pounds pressure for ten minutes was believed to be insufficient for destroying the spores. Thom et al (1919) reported that the Boise strain withstood a pressure of 10 pounds for fifteen minutes or 100°C., for one hour. Fifteen pounds for fifteen minutes in the autoclave destroyed it.

EXPERIMENTAL

Spore suspensions and cultures. The cultures used in these determinations were obtained from Dr. Robert Graham, Department of Animal Pathology, University of Illinois. They were numbered as follows with source: 820-A, isolated from garbage which caused avian botulism in Urbana, type A organism; 854-A, also type A, obtained from the spleen of a calf which died showing all of the characteristic symptoms of botulism: 820-B, a type B organism, isolated from corn ensilage which was known to have caused botulism among cattle in Iroquois County; G-6-B, another type B strain, isolated from oat hay, which, when inoculated into monkeys, produced death with the characteristic symptoms. In producing the spores of *Clostridium botulinum* a brain medium of thinner consistency than that described by Weiss (1921a) was utilized.

Medium. This was made by adding one part of finely macerated sheep's brain to two parts of water. It was then sterilized in an autoclave at 15 pounds pressure for thirty minutes in 250 cc. Pyrex Erlenmeyer flasks, from 125 to 150 cc. of medium in each flask. This medium was used without adjustment to grow the organism and produce the spore suspension since it was found to be practically neutral (pH 6.8 to 7.0). Dickson and Burke reported the use of a medium consisting of a 1 per cent glucose peptic digest of liver broth adjusted to pH 7.3 to 7.5.

Inoculations. Inoculations were made by means of 10 cc. sterile pipettes, 10 cc. of a stock suspension in brain medium

being transferred to each flask. Melted paraffin was then poured into the flask which hardened to give a coating on top of about 1 cm. in thickness. At a later date we tried growing the organisms without covering the culture with paraffin to determine whether a larger number of spores could be produced in the suspension, but have not sufficient data at present to warrant any conclusions. This procedure has been suggested by various investigators for certain of the other pathogenic anaerobes.

The flasks were then incubated at room temperature for approximately 30 days in the dark. To determine the number of spores produced the Torrey anaerobic plate was used. The medium used was a 0.5 per cent glucose agar in which dilutions of 1 to 1000 and 1 to 10,000 were inoculated. A covering of glucose agar was then poured on top and finally a coating of paraffin, colored with Sudan III, as suggested by Northrup to make the colonies stand out in relief. These cultures were incubated from ten days to two weeks and counted. The average number of spores obtained from the dilution plate counts was 3,200,000 per cubic centimeter.

Apparatus. Thermal determinations were made using a DeKotinsky electric bath, with a thermo regulator attachment, turbine agitator, Nujol for the liquid of high boiling point, and Wasserman test tube rack for immersion of tubes. Temperatures of 100°C.; 105°C.; 110°C.; 115°C.; and 120°C; were used in the experimental heating of the organisms.

The cultures used were transferred by means of a sterile pipette from the 250 cc. flasks to sterile, special soft glass tubes (as recommended by Bigelow and Estey (1920), having an inside bore of 7 mm., an outside diameter of 9 mm., and 250 mm. in length), 1 cc. of media to each tube. The tubes were than drawn out, 70 mm. above the top of the medium, to obtain a short capillary neck above the medium. One set was then exhausted to give a vacuum of about 17 mm. and sealed at the capillary portion. The second set was not exhausted but left plugged with cotton and heated as open tubes.

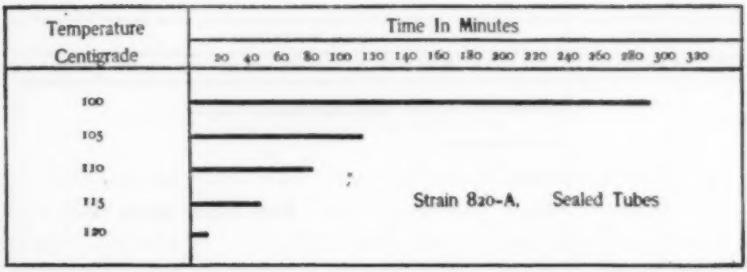
It was found to be troublesome to use the open tubes at temperatures above 100°C., since the medium boiled up in the tubes to the plugs and gave opportunity for too great an error. To

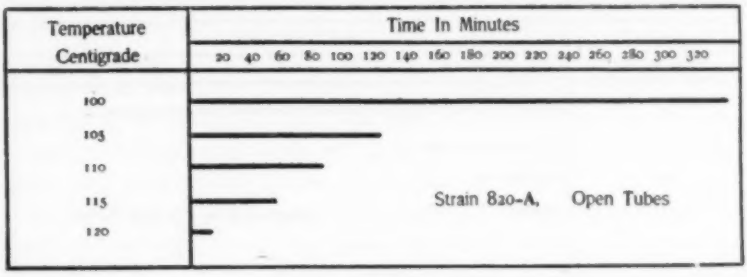
overcome this we drew out the tube in the same manner as for the sealed tubes, and pushed a tight cotton plug down until it was stopped by the capillary constriction. After heating, and immediately as each tube was taken from the bath, it was sealed in a flame to prevent any material which may have been unevenly heated from being drawn down into the tube as it cooled.

The spores used in these tubes were about thirty days old, it being impractical to utilize those exactly thirty days old at all times. The variation in resistances of a few days when the spores were approximately thirty days old was shown by Weiss to be negligible.

Incubation. One tube of each culture was taken out of the oil bath at stated intervals, cooled at once by immersion in cold water, and inoculated into 10 cc. tubes of sterile brain medium, care being taken to mix the spore suspensions with the brain medium. As stated above, the open tubes were sealed immediately and then cooled. The tubes of brain medium were then covered with paraffin to a depth of about 1.5 cm., plugged and incubated at room temperature in the dark. Owing to the time of completion of the thermal determinations, these tubes were incubated over the summer vacation and averaged an incubation period of four months.

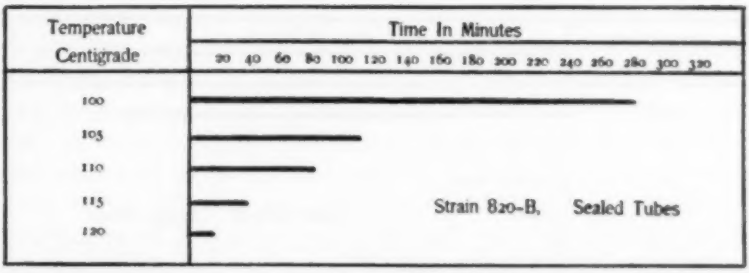
Inoculation. All cultures showing gas formation with the characteristic foul odor were discarded as indicative of active growth and the first one of the series showing no gas formation was taken for further study to ascertain if there had been any growth and toxin formation. One cubic centimeter of this latter culture was fed to a guinea pig through a sterile pipette, and the pig kept under observation for one month. If death with the characteristic symptoms did not ensue in this time the spores were considered to have been destroyed. It was found that our results supported the observations of Weiss, that the spores were progressively injured before death finally ensued. Some pigs inoculated orally, lived for periods of from three to nineteen days, showing that the organisms had been so injured that only a small amount of toxin or perhaps a toxin of low virulence had been produced.

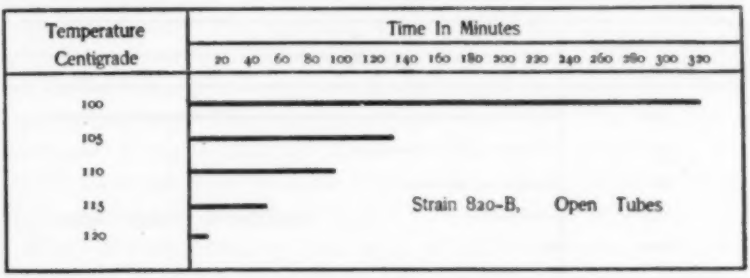




STRAIN 820-A

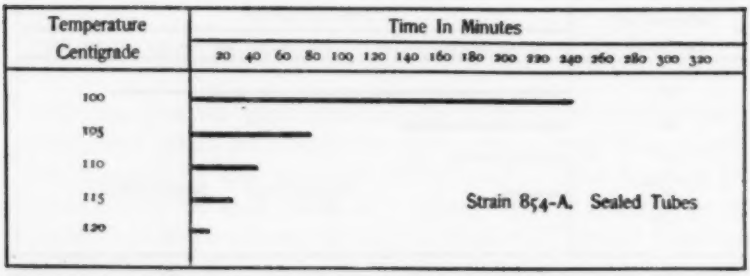
Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.

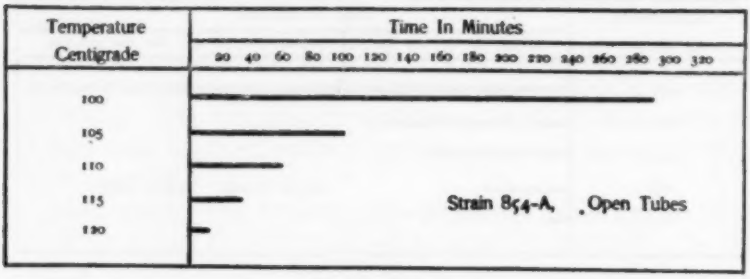




STRAIN 820-B

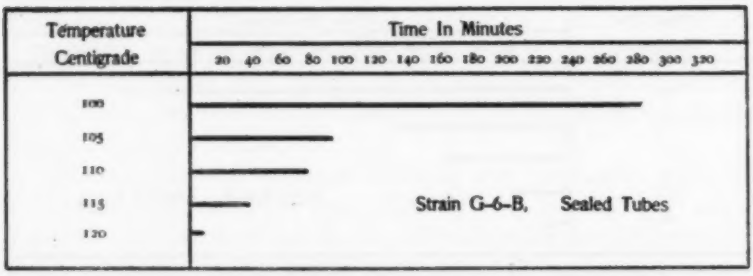
Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.

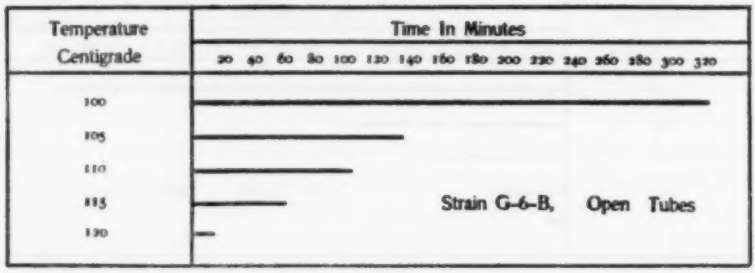




STRAIN 854-A

Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.





STRAIN G-6-B

Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.

DISCUSSION OF RESULTS

From the accompanying tables it will be seen that there is a variation in resistance of different strains of spores under controlled conditions. The most resistant spores were found to be destroyed in less than five hours at 100°C. in the cultures which had been exhausted to obtain a 17 mm. vacuum, and in five and one-half hours in the open tubes. Bigelow and Estey (1920) stated that the time necessary for the tubes to reach 100°C, after being immersed in the bath was on the average 15 seconds since the tubes used were small in diameter, only moderately thick, and completely immersed in the oil bath.

At 105° the cultures showed a variation of thirty minutes in death points obtained, the most resistant being destroyed in one hour and fifty minutes for the tubes exhausted to 17 mm. vacuum. In the open tubes there was also a variation of 30 minutes, the most resistant cultures being killed in two hours and ten minutes. The same conditions hold for 110°C., the cultures varying ten minutes and showing an irregularity in the final death point as determined by injection into guinea pigs. All cultures were found to be destroyed in less than an hour and a half in the sealed tubes. The open tubes showed a greater variation in thermal death time, the most resistant being killed in one hour and forty minutes, while the least resistant one was destroyed in fifty-five minutes. At 115°C. we see less variation in death point in vacuum tubes, all cultures being killed in forty minutes in the sealed tubes. The open tubes required a slightly longer time, the most resistant spores being destroyed in fifty minutes. At 120°C. no cultures survived ten minutes heating but 820-A does show slight toxin formation at the end of five minutes heating. This tube when inoculated into brain medium, incubated three months at 21°C., and fed to a guinea-pig, required nineteen days to cause death. All other cultures caused death of the guinea-pigs in twelve to seventy-two hours.

The variation between the open tubes and those exhausted and sealed to give a vacuum of 17 mm. is small but the time required in the case of the open tubes was always found to be longer.

The change in pH during the process of heating was found to be so small as to be negligible, the tubes having first been soaked over night in cleaning solution. The change in four hours with the tubes not so cleaned was found to average 0.6 by Morrison (unpublished data).

The reaction of our media was found by numerous determinations to average pH 6.8. This was kept in Pyrex flasks and used without adjustment. It was found that brain medium put up in soft glass flasks and kept for periods of twenty-one days or more had an average reaction of about pH 7.4. Either medium could be used to grow our cultures, both apparently being of equal value. With media testing pH 6.8, growth of the organism produced a gradual change to the alkaline side, reaching pH 7.2; with media at pH 7.4, instead of producing greater alkalinity, it, too, reached an average of 7.2. Weiss reports the pH as becoming stable at 7.5 after cultural growth in brain medium.

SUMMARY AND CONCLUSIONS

1. The spores of *Clostridium botulinum* in sealed tubes exhausted to 17 mm. vacuum are destroyed within a period of five hours at 100°C.; within two hours at 105°C.; within one and one-half hours at 110°C.; within forty minutes at 115°C.; and within ten minutes at 120°C.

2. A longer heating period was required to destroy spores of the same age in open tubes than in tubes exhausted and sealed, under the conditions that obtained in this investigation.

3. The death of spores is probably a gradual process. Surviving spores may have their internal mechanism so injured that weak toxins are formed.

REFERENCES

BIGELOW, W. D., AND ESTEY, J. R. 1920 Jour. Inf. Dis., **27**, 602.
BURKE, G. S. 1919a Jour. Amer. Med. Assoc., **78**, 88–92.
BURKE, G. S. 1919b Jour. Bact., **4**, 555–565.
THOM, C. ET AL. 1919 Jour. Amer. Med. Assoc., **73**, 907–912.
WEISS, H. 1921a Jour. Inf. Dis., **28**, 70–92.
WEISS, H. 1921b Jour. Inf. Dis., **29**, 362.

THE FERMENTATION OF ARABINOSE AND XYLOSE BY CERTAIN AEROBIC BACTERIA[1]

E. B. FRED, W. H. PETERSON, AND J. A. ANDERSON

From the Departments of Agricultural Bacteriology and Agricultural Chemistry, University of Wisconsin, Madison

Received for publication August 9, 1922

During a study of the aerobic flora from lake water, silage and vinegar, it was found that many of these organisms attack pentose sugars. In contrast to the acid formers which are described in earlier papers (Fred et al, 1919; 1921), these aerobic organisms ferment the pentose sugars, arabinose and xylose more slowly and produce only a small amount of acid. The total yield of fixed acid is far too small to account for the carbohydrate consumed. Products other than acids must be formed and various experiments were carried out in order to study the products of the aerobic fermentation and the proportion in which they are found.

Six cultures of bacteria representing various groups of the more common aerobic types were used in this study. *Bacillus vulgatus* (Flügge) Trevisan, synonomy *Bacillus mesentericus vulgatus*, was isolated from the mud of Lake Mendota; *Acetobacter sorbose* and *Acetobacter xylinum* were obtained from the American Museum of Natural History, and originally came from Bertrand; while three chromogenic forms, one a rod which answers to the description of *Bacterium herbicola aureum* (Düggeli 1904) and the others two coccus forms designated "Organism A, and B" were isolated from green sweet corn.

Some of the characteristics of these aerobic pentose fermenters are shown in table 1. Aside from the great difference in morphology, these forms are readily distinguishable by definite

[1] This work was supported in part by a grant from the special research fund of the University of Wisconsin. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

TABLE 1

The characteristics of certain aerobic pentose fermenters

NUMBER	BACILLUS VULGATUS	ACETOBACTER SORBOSE	ACETOBACTER XYLINUM	ORGANISM A	ORGANISM B	BACTERIUM HERBICOLA AUREUM
1. Form	Rod	Rod	Rod	Coccus	Coccus	Rod
2. Spores	Present	None	None	None	None	None
3. Flagella	Present	Present	Present	None	None	Present
4. Pigment	Fawn-colored	Light-yellow	Brownish-yellow	Orange	Yellow	Light-yellow
5. Gram stain	Positive	Positive	Positive	Positive	Positive	Negative
6. Catalase test	Very strong	Strong	Fair	Strong	Very strong	Very strong
7. Carbinol test						
Glucose	Strong	Fair	Medium	None	None	None
Arabinose	Strong	Trace	Medium			
Xylose	None	None	None			
8. Gelatin	Liquefied	Not liquefied	Not liquefied	Slow liquefication	Rapid liquefication	Not liquefied
9. Litmus milk	Reduced, soft curd later digestion	Reduced, brown serum zone, soft curd	Reduced, pink serum zone, soft curd	Alkaline, reduced	Alkaline, digestion	Yellow, no change

physiological characters. These differences are perhaps best seen from a comparison of the action of the bacteria in milk and in gelatin. On solid and liquid media, *B. vulgatus* forms a wrinkled membrane which spreads rapidly over the entire surface. *Acetobacter sorbose* on glucose yeast-water agar forms a pale yellow film. In liquid the medium becomes turbid throughout. *Acetobacter xylinum* forms a thick pink to brown membrane on glucose yeast-water agar slopes. The coccus forms A and B produce a raised profuse growth on agar slopes and a decided turbidity in liquid media.

In yeast water solutions the three chromogenic forms ferment glucose, mannose, fructose, sucrose, maltose, lactose, melezitose salicin, mannitol and glycerol with the production of a small amount of fixed acid. Raffinose, alpha-methyl glucoside, dulcitol, inulin and starch are not attacked. Aside from total acidity, the amount of sugar fermented was also determined. In general it was found that *B. herbicola aureum* is the most active fermenter of the three, destroying from 50 to 60 per cent of the sugar in the first ten days. Organism B in turn is more active in the fermentation of sugars than Organism A.

THE FERMENTATION OF ARABINOSE AND XYLOSE BY B. VULGATUS ACETOBACTER SORBOSE AND ACETOBACTER XYLINUM

In preliminary experiments to determine the fermentation of the pentoses by these organisms 25 cc. cultures of 2 per cent sugar medium were grown in 100 cc. Erlenmeyer flasks. The source of the nitrogen was varied. However, no well defined difference in fermentation was noted from the use of beef broth or of peptone broth in place of the yeast water. In general, the yeast water proved slightly superior and hence in all of the quantitative work which follows, this medium was employed.

The methods for measuring the products of fermentation with these aerobic forms were essentially different from those used in the work on acid fermentation of pentoses by Lactobacilli. The fermentation was carried out in a closed system, figure 1, through which carbon dioxide-free air was drawn. The fermentation flask *C* was closed with a two-hole rubber stopper, which

fitted tightly in the neck of the flask (see *F*). Through one hole was passed a long glass tube reaching almost to the surface of the culture solution. The other end of this glass tube carried a cotton plug *A*. Through the second hole in the rubber stopper a short glass tube *B* was passed. The upper end of this tube *B* was closed by means of a piece of rubber tubing, a screw

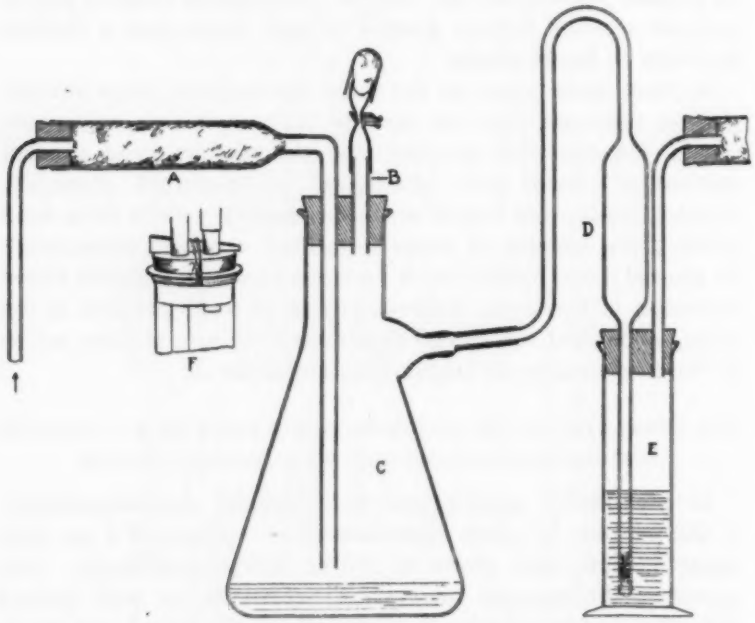


FIG. 1. FLASK FOR THE GROWTH OF AEROBIC BACTERIA AND THE ABSORPTION OF EVOLVED CARBON DIOXIDE

pinch-cock, and a cotton plug, protected by a short inverted test tube. Through tube *B*, samples were removed for analysis. The flask *C* was connected to a 50 cc. pipette, which in turn passed into the alkali flask *E*. This flask contained a solution of 30 per cent KOH. By means of a bottle aspirator, a slow current of CO_2-free air, was drawn through the system of flasks to remove evolved gases. The carbon dioxide was determined

by double titration according to the method of Scales (1920). As a check on the Scales method CO_2 was also determined by the gasometric method of Van Slyke (1917).

The cultures were connected in series and aspirated continuously with a current of CO_2-free air. During the incubation period, usually four weeks, the entire apparatus was kept at 28°C. At irregular intervals the CO_2 receivers were removed and the CO_2 determined. After some preliminary tests, it was found that this kind of fermentation flask if carefully set up may be kept for months without contamination and without any loss of CO_2. No attempt was made to measure gaseous products other than CO_2.

Since all of the organisms showed an appreciable growth in the yeast water alone, each organism was grown in this medium and the carbon dioxide found was deducted from the total gas production on the yeast water plus sugar. All three cultures, *B. vulgatus*, *A. sorbose* and *A. xylinum* break down xylose with the production of large amounts of CO_2. This substance is far in excess of all others and accounts for a large part of the sugar consumed. The data obtained from the fermentation of xylose in yeast water are given in table 2. As compared with the acid fermentation of xylose by Lactobacilli, the most noticeable difference is the large CO_2 production and the absence of acids. Since atmospheric oxygen is utilized in the fermentation process, a direct comparison cannot be made between the total weight of products formed and the weight of sugar consumed.

Gruner and Fraser (1921) in their study of the *Bacillus mesentericus* group and allied organisms, called attention to the action of this group on sugar media. They found that acid formation from glucose usually reaches a maximum by the second or third day and on longer periods of incubation fails to increase. Results somewhat similar to those of Gruner and Fraser were obtained with *B. vulgatus*. At first there was a small amount of acid, which in the older cultures disappeared almost completely.

Of special interest is the production of acetone and ethyl alcohol from xylose by both *B. vulgatus* and *A. xylinum*. The acetone organisms which have been isolated and studied

B. macerans (Schardinger, 1905), *B. granulobacter pectinovorum* (Speakman, 1920), *B. acetoethylicum* (Northrop, Ashe and Senior, 1919), *B. butylicus* (Ricard, 1919) are facultative anaerobic rod forms. In contrast to the organisms described in this paper these acetone forming bacteria produce acid, usually acetic or butyric, together with the neutral substances, acetone and alcohol. A recent paper by Berthelot and Ossart (1921) gives the results of acetone production by 217 cultures of bacteria isolated from soil, water and manure. Their data are substantially in agreement with the figures of table 2.

TABLE 2

Total fermentation products from xylose

Xylose at beginning 1.89 per cent. Calculated for 100 cc. of culture.

PRODUCTS	B. VULGATUS	A. SORBOSE	A. XYLINUM
	gm.	gm.	gm.
Carbon dioxide			
From xylose and yeast water	2.3990	2.1702	2.2223
Yeast water alone	0.8068	0.3457	0.8437
From xylose	1.3922	1.8245	1.3786
Ethyl alcohol	0.0784	0.0	0.3698
Acetone	0.0088	0.0	0.1847
Total products	1.4794	1.8245	1.9331
Xylose fermented	1.4007	1.6090	1.2530

The two organisms *A. sorbose* and *A. xylinum*, behaved somewhat differently from the description given by Brown (1886) and by Bertrand (1904). The general cultural characteristics answer to those given in the original description, but the products of fermentation were markedly different. Instead of forming arabonic acid from arabinose and xylonic acid from xylose, as described by the original investigators, the main products of fermentation were found to be carbon dioxide by *A. sorbose* and carbon dioxide, ethyl alcohol and acetone by *A. xylinum*. The formation of these two neutral products was totally unexpected and to be sure that it was not due to contamination, this experiment was repeated several times and always with the same results. *A. xylinum* produces acetone and alcohol from xylose in

large amounts. If calculated in percentage of the sugar consumed, the acetone is equivalent to 10.8 per cent, the alcohol to 18.8 per cent. These values are of special interest for comparison with those recorded in papers by Speakman (1920) for *B. granulobacter pectinovorum* and by Artzberger et. al (1920) for the acetone organism, *B. acetoethylicum*. The percentages are approximately of the same order as those reported by these investigators.

EFFECT OF AGE ON ACETONE PRODUCTION

In preliminary tests it was found that the age of the culture influences the amount of acetone. The next experiment was

TABLE 3

Effect of age of cultures on acetone and alcohol production

Calculated for 100 cc. of culture.

PRODUCT	AGE OF CULTURE	B. VULGATUS	A. XYLINUM
	days	*gm.*	*gm.*
Acetone	6	0.0902	0.0651
Acetone	20	0.0024	0.0843
Ethyl alcohol	6	0.1122	0.1325
Ethyl alcohol	20	0.0147	0.1472
Xylose fermented in twenty days		90.26 per cent	70.92 per cent

undertaken in an attempt to find out the amount of acetone at different ages of the cultures, *B. vulgatus* and *A. xylinum*. Table 3 shows the values obtained for acetone and alcohol at varying ages. These two cultures are strikingly different. *B. vulgatus* forms large amounts of acetone and alcohol in young cultures and later shows a decided decrease in these two substances, while *A. xylinum* shows only a small amount of acetone and alcohol in young cultures but increases continually to a large amount in old cultures.

THE FERMENTATION OF ARABINOSE AND XYLOSE BY B. HERBICOLA AUREUM AND ORGANISMS A AND B

Because of the frequent occurrence of these three chromogenic types on green plants, their action on the pentose sugars is of particular importance. Although widely distributed in nature and easily isolated, these three forms fail to exhibit any striking physiological characteristics. No doubt these, or closely related organisms, inhabitants of living plant tissue, play an important rôle in nature.

TABLE 4

Total fermentation products from pentoses

Calculated for 100 cc. of culture.

PRODUCTS	XYLOSE AT BEGINNING 1.9765 PER CENT			ARABINOSE AT BEGINNING 1.8970 PER CENT
	Organism A	Organism B	*B. herbicola* aureum	*B. herbicola* aureum
	gm.	*gm.*	*gm.*	*gm.*
Carbon dioxide				
From sugar and yeast water	0.5810	0.6800	1.6667	2.3257
From yeast water alone			0.3946	0.3946
From sugar			1.2721	1.9311
Sugar fermented	0.6900	0.6850	1.5654	1.6328

The apparatus used and methods of study described in the first part of this paper were followed. After thirty days the cultures were analyzed and the results shown in table 4. These two coccus forms are slow fermenters of xylose destroying about 30 per cent, but *B. herbicola aureum* attacks the pentose sugars much more vigorously. In thirty days about 79 per cent of the xylose and 81 per cent of the arabinose was fermented. The products of fermentation are almost entirely CO_2 and a small amount of acid. From *B. vulgatus* 0.076 gram of non-volatile acid calculated as lactic was obtained for 100 cc. of medium.

SUMMARY

This report deals with the fermentation of the pentose sugars by certain aerobic bacteria, viz., *B. vulgatus*, *A. sorbose*, *A. xylinum*, *B. herbicola aureum* and two yellow coccus forms designated Organism A and B. The rate of fermentation of xylose, and in certain cases arabinose, by these aerobic forms is much slower than is noted with the facultative anaerobic bacteria.

The products of the fermentation of the pentose sugars depends upon the kind of organism and the ratio of the products on the age of the culture. *B. vulgatus* ferments xylose with the production of acetone, ethyl alcohol, carbon dioxide and a small amount of fixed acids. As the culture becomes older the ratio of acetone and ethyl alcohol to carbon dioxide decreases. The maximum amount of acetone and alcohol is found about the sixth day after inoculation.

In the case of *A. xylinum* an increase in the age of the culture is accompanied by an increase in the yield of acetone and alcohol. Although in different proportions the substances obtained in the breaking down of xylose, *A. xylinum* are the same as noted with *B. vulgatus*. *A. sorbose* *B. herbicola aureum* and the two coccus forms ferment the pentoses slowly and the chief end product is carbon dioxide and a trace of acid.

It may be concluded that in nature many of the commonly occurring aerobic forms of bacteria attack the pentose sugars and form as the chief end products, neutral substances such as acetone and ethyl alcohol, and carbon dioxide.

An apparatus for conducting the fermentations under aerobic conditions is described.

REFERENCES

ARZBERGER, C. F., PETERSON, W. H., AND FRED, E. B. 1920 Jour. Biol. Chem., **44**, 465.

BERTHELOT, A., AND OSSART, E. 1921 Compt. Rend., **173**, 792.

BERTRAND, G. 1904 Ann. de Chimie et de Physique, 81 Ser., **3**, 181.

BROWN, A. J. 1886 Chem. Soc. Jour. (London) (Trans.), **49**, 432.

DÜGGELI, MAX 1904 Centr. Bakt. 2te Abt., **13**, 56, 198.

FRED, E. B., PETERSON, W. H., AND ANDERSON, J. A. 1921 Jour. Biol. Chem., **48**, 385.

FRED, E. B., PETERSON, W. H., AND DAVENPORT, A. 1919 Jour. Biol. Chem. **39**, 347.
GRUNER, O. C., AND FRASER, J. R. 1921 Jour. Inf. Dis., **10**, 210.
NORTHROP, J. H., ASHE, L. H., AND SENIOR, J. H. 1919 Jour. Biol. Chem., **39**, 1.
RICARD, E. 1919 Jour. Soc. Chem. Ind., (London), **38**, 787.
SCALES, F. M. 1920 Science, n.s., **51**, 214.
SCHARDINGER, F. 1905 Centr. Bakt., 2te., Abt. **14**, 772.
SPEAKMAN, H. B. 1920 Jour. Biol. Chem., **41**, 319.
VAN SLYKE, D. D. 1917 Jour. Biol. Chem., **30**, 347.

THE RELATION BETWEEN CHEMICAL COMPOSITION OF PEPTONES AND HYDROGEN SULPHIDE PRODUCTION BY BACTERIA

F. W. TILLEY

From the Biochemic Division, Bureau of Animal Industry, United States Department of Agriculture

Received for publication August 19, 1922

In a paper recently published in this journal (Tilley, 1923) the writer showed that variations in hydrogen sulphide production by bacteria in lead acetate agar may be due on the one hand to differences in the hydrogen sulphide producing power of different strains of the same organism and on the other hand to the use of different varieties of peptone in the lead acetate agar. As explained in that paper the writer had expected to find that the value of the various peptones for H_2S production depended upon the amount of cystin present, just as the value of peptones for indol production depends upon the tryptophan content (Tilley, 1921). Preliminary experiments, however, indicated that the problem was more complicated than at first supposed so further work was undertaken, the results of which are reported in the present paper.

EXPERIMENTAL WORK

In view of the great complexity of the composition of peptones it was not considered advisable to attempt to make complete and exact analyses of the various peptones used. For the purposes of the work here reported it was thought sufficient to ascertain roughly the approximate amounts of "unoxidized," "partly oxidized" and "oxidized" sulphur in the six peptones employed in the work reported in the previous paper.

The term "unoxidized sulphur" will be used in this paper to denote sulphur as it occurs in proteins and protein derivatives

loosely combined with carbon and hydrogen. Upon heating with caustic alkali hydrogen sulphide is liberated and as it is detected by means of lead acetate solution or lead acetate paper this form of sulphur is often called "lead blackening sulphur." Cystin is an example of this form of sulphur. The term "partly oxidized sulphur" will be used to denote sulphur combined in such a form that upon distillation with phosphoric acid sulphur dioxide is liberated. The term "oxidized sulphur" will be used to denote sulphur as it occurs in sulphates or other similar compounds.

Unoxidized sulphur was estimated in the following manner: 5 cc. of a strong (1:1) solution of potassium hydroxide were

TABLE 1

Relation between chemical composition of peptones and H_2S production by bacteria

PEPTONE NUMBER	UNOXIDIZED SULPHUR	PARTLY OXIDIZED SULPHUR	OXIDIZED SULPHUR	H_2S BY BACTERIA
1	+	+	+	+
2	−	+++	+++	+++
3	++	−	−	−
4	++	++	−	++
5	+++	+	+++	++
6	−	+++	+++	+++

− = none; + = small amount; ++ = moderate amount; +++ = large amount.

mixed with an equal quantity of a filtered 3 per cent solution of peptone in water or in 20 per cent hydrochloric acid; a few drops of a 10 per cent aqueous solution of lead acetate were added and the mixture boiled vigorously. The relative amounts of unoxidized sulphur present were estimated roughly by the amount of blackening which resulted.

The estimation of partly oxidized sulphur was made by Mr. R. R. Henley of this Division, to whom I am also much indebted for advice and assistance so far as the chemical aspects of this work are concerned. The details of the method employed may be found in Bureau of Chemistry Bulletin No. 107 (1908). Oxidized sulphur was estimated by precipitation with barium chloride in an acid solution.

The relation between chemical composition of the various peptones, as shown by the previously described tests, and hydrogen sulphide production by bacteria in lead acetate agar containing these peptones is shown in table 1. It should be clearly understood that the relative amounts indicated are only rough approximations. In order to avoid any suggestion that the differences shown represent exact quantitative results arbitrary symbols are employed instead of any statement of amounts.

The results shown in table 1 indicated that partly oxidized sulphur is the form of sulphur which yields the largest amount of hydrogen sulphide. Further experiments were, however,

TABLE 2

Relative values of oxidized and partly oxidized sulphur for H_2S production

ORGANISM AND STRAIN	H_2S PRODUCTION BY BACTERIA IN MEDIA CONTAINING		
	No added sulphur	Ammonium sulphate	Sodium thiosulphate
Proteus 4	+	+	+++
Bact. suipestifer 416	+	+	+++
Bact. suipestifer 420	+	+	+++
Bact. enteritidis G 4	+	+	+++
Bact. typhosum A	+	+	++
Bact. typhosum C	+	+	++
Bact. typhosum D	−	−	−

− = no H_2S; + = small amount; ++ = moderate amount; +++ = large amount. Results shown are after twenty-four to forty-eight hours incubation.

undertaken to determine more exactly the relative value of the different forms of sulphur for hydrogen sulphide production. In these experiments various sulphur compounds were added to lead acetate agar and observations made upon the relative amounts of hydrogen sulphide produced by various bacteria.

In the first place an attempt was made to show the relative values of oxidized sulphur in the form of sulphate and partly oxidized sulphur in the form of thiosulphate. These compounds were added to lead acetate agar prepared according to the directions of Jordan and Victorson (1917) except that the reaction was adjusted to pH 7.2 instead of to +1 to phenolphthalein. The amount of sulphate or thiosulphate added was 0.25 per cent.

The peptone used in the preparation of the lead acetate agar was peptone 1 (see table 1). The results are given in table 2.

The results shown in table 2 indicate that oxidized sulphur in the form of sulphate does not yield any appreciable amount of hydrogen sulphide while partly oxidized sulphur in the form of thiosulphate seems to be especially suitable for hydrogen sulphide production by bacteria.

TABLE 3

Comparative effect of different compounds containing partly oxidized sulphur

ORGANISM AND STRAIN	H_2S PRODUCTION BY BACTERIA IN MEDIA CONTAINING		
	No added sulphur	Sodium thiosulphate	Sodium sulphite
Bact. suipestifer 360	−	−	+++
Bact. suipestifer 416	−	+++	+
Bact. coli C	−	−	+
Bact. cloacae T 2	−	−	+
Proteus 4	−	+++	+
Proteus 11	−	+	−
Bact. typhosum A	−	+++	+++
Bact. typhosum K 110	−	−	++
Bact. dysenteriae Y	−	−	+
Bact. dysenteriae Shiga	−	−	−
Paratyphoids	−		
A. K A 28	−	−	++
B. K B 31	−	+++	+++
C. K C 1	−	+++	+++

− = no H_2S; + = small amount; ++ = moderate amount; +++ = large amount.

In the remaining experiments instead of following the directions of Jordan and Victorson, lead acetate agar was prepared by adding lead acetate solution to ordinary beef infusion agar containing 1 per cent of peptone 1. In the experiment shown above in table 3, sodium thiosulphate or sodium sulphite was added to this medium in the proportion of 0.25 per cent.

The results shown above indicate that, while both sodium thiosulphate and sodium sulphite readily yield hydrogen sulphide, sodium thiosulphate, on account of clearer distinctions between organisms and strains, would give the better results when used

in a culture medium intended for diagnostic purposes to distinguish between different species and strains of bacteria.

In table 4 there are shown the results of an experiment with representatives of the two classes of organic compounds containing sulphur designated by Hawk (1918, p. 108) as unoxidized and oxidized. Cystin was selected as containing unoxidized organic sulphur and taurin as containing oxidized organic sulphur.

TABLE 4

Comparative value of different organic sulphur compounds for H_2S production

ORGANISM AND STRAIN	H_2S PRODUCTION BY BACTERIA IN MEDIA CONTAINING		
	No added sulphur	Cystin	Taurin
Bact. suipestifer 360	–	+	–
Bact. suipestifer 416	–	++	–
Bact. coli C	–	+++	–
Bact. cloacae T 2	–	++	–
Proteus 4	–	+++	–
Proteus 11	–	+++	–
Bact. typhosum A	–	++	–
Bact. typhosum K 110	–	+	–
Bact. dysenteriae Y	–	+	–
Bact. dysenteriae Shiga	–	+	–
Paratyphoids	–		
A. K A 28	–	++	–
B. K B 31	–	+++	–
C. K C 1	–	+++	–

– = no H_2S; + = small amount; ++ = moderate amount; +++ = large amount.

The technique employed was similar to that used in experiment III except that the amount of cystin used was 0.1 per cent and special technique was employed in adding it to the culture medium. The cystin was partly dissolved and partly suspended in N/10 HCl and after sterilization in the autoclave was added to the culture medium with a sterile pipette. Just previous to this there was added enough sterile N/10 NaOH to neutralize the N/10 HCl in which the cystin was carried, thus leaving the reaction of the culture medium as far as possible unchanged.

The results given in table 4 indicate that taurin is not utilized by bacteria for H_2S production while cystin gives rise to an abundant production of H_2S, so much so that differences between the various species and strains are largely obliterated. This, and the fact that organisms usually classed as "lead negative" are rendered "lead positive," make it inadvisable to use cystin to correct deficiencies in the sulphur content of peptones used in lead acetate agar.

The results of table 4 taken together with those of table 1 bring up a very interesting question. In table 4 we see that unoxidized sulphur in the form of cystin yields a large amount of H_2S and yet in table 1 we see that although peptone 3 contains apparently as much unoxidized sulphur as any of the peptones except no. 5, no appreciable amount of H_2S is formed in lead acetate agar made with peptone 3.

The most plausible explanation of this discrepancy would seem to be that the unoxidized sulphur of the various peptones is not all free cystin.

In order to test this explanation the six peptones previously employed were examined for free cystin in the following manner, the technique employed being based on that given by Hawk (1918, p. 87) for the separation of cystin formed by the action of hydrochloric acid on wool; 5 grams of peptone were dissolved in 25 cc. of 20 per cent hydrochloric acid, the solution being kept cool to avoid hydrolytic action as far as possible, and the resultant solution filtered. To this filtered solution sodium acetate was added in excess, as shown by a negative reaction for mineral acid with Congo red. Whatever precipitate was formed was separated out by filtration through a hardened filter paper and then dissolved in hot dilute (5 per cent) hydrochloric acid. Then this dissolved precipitate, and also the filtrate from the original solution, were tested for unoxidized sulphur by the method already given in the first part of the paper. Free cystin being insoluble in acetic acid should appear in the precipitate while any unoxidized sulphur found in the filtrate would be regarded as something other than free cystin. The results are shown in table 5.

The results shown below indicate that none of the peptones

except no. 5 contain any really appreciable amount of free cystin. The presence of cystin in no. 5 is no doubt the reason for the results shown in table 1 where it is seen that although peptone 5 contains very little partly oxidized sulphur it yielded a fairly large amount of hydrogen sulphide. The fact that the cystin served to bring up the amount of hydrogen sulphide only to a moderate degree is presumably due to its relative insolubility. In spite of the large amount in the peptone only a small amount was dissolved by the culture media.

TABLE 5

Relative amounts of free cystin and other unoxidized sulphur in peptones

PEPTONE NUMBER	UNOXIDIZED SULPHUR	
	Precipitate	Filtrate
1	None	Present
2	None	Present
3	None	Present
4	A trace	Present
5	Present	Present
6	None	Present

DISCUSSION

It is interesting, in the first place, to note that the results shown in tables 2, 3 and 4 agree very well with those reported by Myers (1920) and by Sasaki and Otsuka (1912). These investigators worked with various sulphur compounds in fluid media containing no peptone but their results correspond very closely to those obtained by the writer with the same compounds in lead acetate agar.

It seems evident from the experimental results herein reported that commercial peptones contain unoxidized, partly oxidized and oxidized sulphur in varying proportions. On account of differences in the amount and availability of these various forms of sulphur in the peptones it is inevitable that variable results should be obtained with lead acetate agar made with the different peptones unless precautions are taken to prevent such results.

Variable results could be avoided by testing the peptones

employed, either by chemical methods similar to those described in this paper, provided the necessary apparatus is available, or by actual trial in media with known strains of known organisms.

The use of sodium thiosulphate as a usual ingredient of lead acetate agar would, however, obviate the necessity for testing the peptone employed and in the writer's judgment, the results obtained would be entirely comparable with those usually obtained with the best peptones. It is quite possible that with organisms other than those used by the writer, or with different strains of the same organisms, this might not hold true but on the other hand the use of a poor peptone will give wholly misleading results, and even with the best peptones there may be variations in the composition of different samples and consequent variations in the results obtained.

It is therefore recommended that sodium thiosulphate be used as a regular ingredient of lead acetate agar. If used in a synthetic medium containing no other source of sulphur it would no doubt give clearer distinctions between different organisms and strains than in a peptone medium. But so far as the writer is aware, there is no synthetic medium which will support as vigorous growth by as many different organisms as the usual peptone media.

In the absence of such a synthetic medium it seems advisable to use lead acetate agar prepared according to the directions of Jordan and Victorson (1917) except for the following changes: the use of 1 per cent instead of 3 per cent of peptone, adjustment of reaction by the hydrogen ion method instead of by titration, and the addition of sodium thiosulphate. The smaller amount of peptone is sufficient to support the growth of bacteria and would lessen possible interference by sulphur compounds already present in the peptone. The writer has in his own work adjusted the reaction to pH 7.2 and used 0.25 per cent of sodium thiosulphate.

CONCLUSIONS

1. Commercial peptones have been shown to contain unoxidized, partly oxidized and oxidized sulphur in varying proportions.

2. Bacteriological tests with media containing various sulphur compounds showed that no hydrogen sulphide was liberated by bacteria from compounds containing oxidized sulphur while it was given off freely from those containing partly oxidized sulphur.

Unoxidized sulphur in the form of cystin yielded an abundance of hydrogen sulphide but experimental evidence indicated that the unoxidized sulphur of commercial peptones may consist largely of some compound, or compounds, other than cystin and not utilized by bacteria for the production of hydrogen sulphide.

3. On account of the qualitative and quantitative differences in the sulphur content of the various commercial peptones, there are resulting variations in hydrogen sulphide production by bacteria in media containing these peptones.

4. To insure uniform results in testing hydrogen sulphide production by bacteria in lead acetate agar the peptone used should be suitable for the purpose, as shown by chemical or bacteriological tests; or, preferably, sodium thiosulphate should be used as an ingredient of the lead acetate agar.

REFERENCES

Bureau of Chemistry Bulletin No. 107 1908 Official and Provisional Methods of Analysis. Association of Official Agricultural Chemists, p. 187.

Hawk, P. B. 1918 Practical Physiological Chemistry. 6th edition.

Jordan, E. O., and Victorson, Ruth 1917 Differentiation of the paratyphoid enteritidis group. II. Lead acetate agar. Jour. Inf. Dis., **21**, 554–555.

Myers, J. T. 1920 The production of hydrogen sulphide by bacteria. Jour. Bact., **5**, 231–252.

Sasaki, T., and Otsuka, I. 1912 Experimentelle Untersuchungen über die Schwefelwasserstoffentwicklung der Bakterien aus Cystin und sonstigen Schwefelverbindungen. Biochem. Ztschr., **39**, 208–215.

Tilley, F. W. 1921 Influence of peptone on indol formation by *Bacillus coli*. Amer. Jour. Pub. Health, **11**, 834–836.

Tilley, F. W. 1923 Variations in hydrogen sulphide production by bacteria. Jour. Bact., **8**, 115.